Positive and negative modulation of the transactivation potential of the transcription factor c-Myb

- novel regulatory mechanisms for a classical proto-oncoprotein -

Thesis submitted for the degree of *Philosophiae Doctor*

by

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ABBREVIATIONS

ADA	Adenosine deaminase
Ada2	Transcriptional Adaptor 2
Adora2B	Adenosine receptor 2B
AMP	Adenosine monophosphate
AMV	Avian myeloblastosis virus
APL	Acute promyelocytic leukemia
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma protein 2
BS69	ZMYND11; zinc finger, MYND domain containing 11
C/EBP	CCAAT/enhancer-binding protein
C/H2	Cysteine/histidine-rich domain 2 (PHD finger)
CASP8AP2	Caspase8-associated protein 2
CB	Cajal bodies
CBP	CREB-binding protein
CD13/APN	Aminopeptidase N
CD4	Cluster of differentiation molecule 4
CD8	Cluster of differentiation molecule 8
c-Fos	v-fos FBJ murine osteosarcoma viral oncogene homolog
ChIP	Chromatin immunoprecipitation
c-Jun	v-jun avian sarcoma virus 17 oncogene homolog
c-kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue
c-Maf	v-maf musculoaponeurotic fibrosarcoma oncogene product homologue
cMGF	Chicken myelomonocytic growth factor
c-myc	v-myc myelocytomatosis viral oncogene homolog
CoIP	Co-immunoprecipitation
CREB	cAMP-responsive element-binding protein
c-Ski	v-ski Sloan-Kettering sarcoma viral oncogene homolog
CtBP	C-terminal binding protein
Daxx	Death-associated protein 6
DBD	DNA-binding domain
DUB	Deubiquitinating enzyme
E1	Ubl activating enzyme
E2	Ubl conjugating enzyme
E26	Avian leukemia virus E26
E3	Ubl ligase
Elk-1	ETS domain-containing protein
EMSA	Electrophoretic mobility shift assay
ENU	*N*-ethyl-*N*-nitrosourea
env	Gene that codes for the viral envelope protein
ER	Estrogen receptor
Ets-1	v-ets erythroblastosis virus E26 oncogene product homolog 1
EVES	Motif within c-Myb NRD
FAETL	Motif within c-Myb NRD
Fat10	Ubiquitin D (Ubl)
FGF-2	Fibroblast growth factor 2

FLASH	FLICE-associated huge protein
gag	Gene that codes for the viral core proteins
Gal4	Yeast activator of galactose-inducible genes
Gam 1	Gallus anti morte 1 from chicken adenovirus type 1
Gas41	Glioma-amplified sequence 41
GATA-3	GATA binding protein 3
GBX2	Gastrulin brain homeobox 2
GR	Glucocorticoid receptor
GTP	Guanosine triphosphate
HAT	Histone acetyl transferases
HDAC	Histone deacetylase
HECT	Homologous to E6-AP C-terminus (ubiquitin E2-interacting domain)
HIPK2	Homeodomain interacting protein kinase 2
HLR	Heptad leucine repeat
HSF	Heat-shock factor
HTH	Helix-turn-helix
IL-6	Interleukin 6
ISG15	Interferon-stimulated gene, 15 kD (Ubl)
IκBα	Inhibitor of NFκB
JNK	c-Jun N-terminal kinase
KIX	CBP/p300 motif interacting with CREB and c-Myb
LZ	Leucine zipper
MafB	v-maf musculoaponeurotic fibrosarcoma oncogene product homolog B
MAPK	Mitogen-activated protein kinase
Mcm4	Mini chromosome maintenance 4
Mdm2	Mouse double minute 2 homolog
Mim-1	Myb-induced myeloid protein-1
MITF	Microphthalmia-associated transcription factor
MRE	Myb recognition/responsive element
MSA	Multiple sequence alignment
mSIN3A	Mammalian SIN3 homolog A
N-CoR	Nuclear receptor co-repressor
NDSM	Negatively charged amino acid-dependent sumoylation motif
Nedd8	Neural precursor expressed developmentally down-regulated protein 8 (Ubl)
NEMO	IκB kinase-regulator
NF-M	Nuclear factor M
NMR	Nuclear magnetic resonance
NOTCH1	Notch homolog 1
NR	Nuclear receptor
NRD	Negative regulatory domain
Nup-358	Nucleoporin, 358 kDa
Pax-5	Paired box-5
Pc2	Polycomb protein 2
PCNA	Proliferating cell nuclear antigen
PDSM	Phosphorylation-dependent sumoylation motif
PIAS	Protein inhibitor of activated STATs
PIC	Pre-initiation complex
PLZF	Promyelocytic leukemia zinc finger
PML	Promyelocytic leukemia gene product
PML-NB	PML nuclear body

pRb	Retinoblastoma susceptibility protein
$R_1R_2R_3$	Myb repeat 1, 2, 3
RAG-2	Recombination activating gene 2
RanBP2	Ran-binding protein 2
RanGAP1	Ran GTPase activating protein 1
RARα	Retinoic acid receptor alpha
RE	Recognition/responsive element
RFP	Ret finger protein
RING	Really interesting new gene (ubiquitin E2-interacting domain)
Rsc	Remodel the structure of chromatin
SAE1/2	SUMO-1 activating enzyme subunit 1/2
SANT	Swi3, Ada2, NcoR and TFIIIB (Histone-interacting domain)
SBM/SIM	SUMO-binding motif/SUMO-interacting motif
SC	Synergy control
SCF	Synergy control factor
SENP	Sentrin-specific protease
SF	Synergy factor
SF-1	Steroidogenic factor 1
SMAD4	SMAD family member 4
SMARCC1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin
SMRT	silencing mediator of retinoid and thyroid receptors
Sp 1	Specificity protein 1
Sp100	Speckled, 100 kDa (nuclear body protein)
SPRING	Siz/PIAS RING
SRF	Serum response factor
SUMO	Small ubiquitin-related modifier
TAB2	TAK1 (MAPKKK)-binding protein 2
TAD	Transactivation domain
TAF	TBP associated factor
T-ALL	T cell acute lymphoblastic leukemia
TBP	TATA-binding protein
TCF-4	T cell factor-4
TCR	T cell receptor
TDG	Thymine DNA glycosylase
TF	Transcription factor
TFIIX	Transcription factor IIX (X = A, B, C, D,E, F or H)
TIF1β	Transcription intermediary factor 1-beta
tom-1	Target of myb 1
TOPIIα	DNA topoisomerase IIα
TOPORS	Topoisomerase I binding, arginine/serine-rich
TP/CR	Threonine-proline-rich/conserved region
TRAF7	TNF receptor-associated factor 7 (E3 ubiquitin ligase)
Ubc9	Ubiquitin conjugating enzyme 9
Ubl	Ubiquitin-like protein
UBM/UIM	Ubiquitin-interacting motif/Ubiquitin-binding motif
ULP	Ubl-specific protease
WRN	Werner Syndrome helicase
Y2H	Yeast two-hybrid
YopJ	Yersinia enterocolitica effector; SUMO-protease homologue

Bases

A	adenine
C	cytosine
G	guanine
T	thymine
U	uracil
R	A or G
Y	C or T
M	A or C
K	G or T
S	C or G
W	A or T
H	A, C or T
B	C, G or T
V	A, C or G
D	A, G or T
N	A, C, G or T

Amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate
E	Glu	Glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
X		Any amino acid
ψ		Hydrophobic amino acid

LIST OF PAPERS

Paper I

Sæther T, Berge T, Ledsaak M, Matre V, Alm-Kristiansen AH, Dahle Ø, Aubry F, Gabrielsen OS. (2007) The chromatin remodeling factor Mi-2α acts as a novel co-activator for human c-Myb. *J Biol Chem*, **282**, 13994-14005.

Paper II

Alm-Kristiansen AH, Sæther T, Matre V, Gilfillan S, Dahle Ø, Gabrielsen OS. (2008) FLASH acts as a co-activator of the transcription factor c-Myb and localizes to active RNA polymerase II foci. *Oncogene*, accepted.

Paper III

Molværsmyr AK, Sæther T, Lorenzo PI, Kvaløy H, Matre V, Gabrielsen OS. (2007) SUMO-conjugation of the transcription factor c-Myb controls cooperative behaviour and induces an activator-to-repressor switch in the negative regulatory domain. Manuscript.

Paper IV

Sæther T, Alm-Kristiansen AH, Troye Pettersen LK, Gabrielsen OS. (2007) A functional SUMO-binding motif in the transactivation domain of c-Myb regulates its activity. Manuscript.

1. INTRODUCTION

The human genome consists of approximately 20-25.000 genes[1], which is much less than first anticipated (80-140.000; [1, 2]). Even smaller than this is the number of genes expressed in a given cell at a given time. The differential expression of genes constitutes the foundation of the cellular diversity seen in higher multicellular organism. The mRNA from genes expressed in a cell, a tissue, an organ or an organism makes up what is referred to as the transcriptome [3]. As the cell goes through the cell cycle, the set of genes transcribed is changing continuously, both in time and amplitude. Different gene programs are turned on and off depending on whether the cell is proliferating or differentiating, responding to hormones or cytokines, whether it is secreting, engulfing or migrating, or whether it is entering apoptosis.

This fine tuned orchestration of gene expression is governed by proteins capable of integrating cellular signals and transmitting them to the general transcription machinery. These proteins are called transcription factors, and this thesis focuses on one such transcription factor, namely the proto-oncoprotein c-Myb.

1.1 TRANSCRIPTION FACTORS

A huge variety of proteins in the cell participates in regulating transcription. These proteins range from being factors residing in the cytoplasm, as the last link in a signal transduction pathway, to the RNA polymerase catalyzing the actual polymerization of the ribonucleotides. In between these two examples we find proteins capable of translocating to the nucleus carrying extranuclear signals and enzymes transmitting chemical signals through post-translation modifications. Within the nucleus there are factors involved in sensing and transmitting information about damaged DNA. We have the co-factors, both repressors and activators, which are able to modulate the activity of other transcription factors through protein-protein interactions or chemical modification. We find chromatin remodelling factors and histone modification enzymes, the general transcription machinery with the general transcription factors, and the mediator complex. Finally, we come to the sequence-specific transcription factors capable of recognizing specific DNA sequences in gene promoters, enhancers and silencers. Today more than 2000 human proteins have been classified as transcription factors. The c-Myb protein is an example of a sequence-specific transcription factor. However, through the introduction and the papers in this thesis, it will

[1] www.ncbi.nlm.nih.gov/genome/guide/human

become evident that c-Myb interacts with, and is regulated by, proteins representing several of the above mentioned classes.

Because of the diversity of transcription factors, attempts have been made to try to classify them; by structure [4], by function [4, 5] and by regulation [6]. The Myb family is a good example of transcription factors that have been classified based on structure. The evolutionary conserved DNA-binding domain (DBD; thoroughly described in chapter 1.2.2.1) has been used to group the members of the Myb-family. Proteins having such a DBD are found in all eukaryotes, where they are most numerous in plants with over 100 family members in *Arabidopsis thaliana* [7] (reviewed in [8-10]). Although similar in DNA-binding properties, the different Myb proteins are not regulated in the same way, do not regulate the same target genes, and do not play the same biological role.

As mentioned, gene expression is tightly regulated to meet the cells physiological needs. This is mainly achieved by regulating the activity and availability of different transcription factors. The regulatory pattern of the sequence-specific transcription factors has therefore been used in an attempt to categorize the them ([6]; Fig 1). This has divided the transcription factors into two major classes: the regulatory (conditionally) and the constitutively active factors. The regulatory factors are subdivided into developmentally regulated factors, active only in certain cell types, and signal-dependent factors, that are

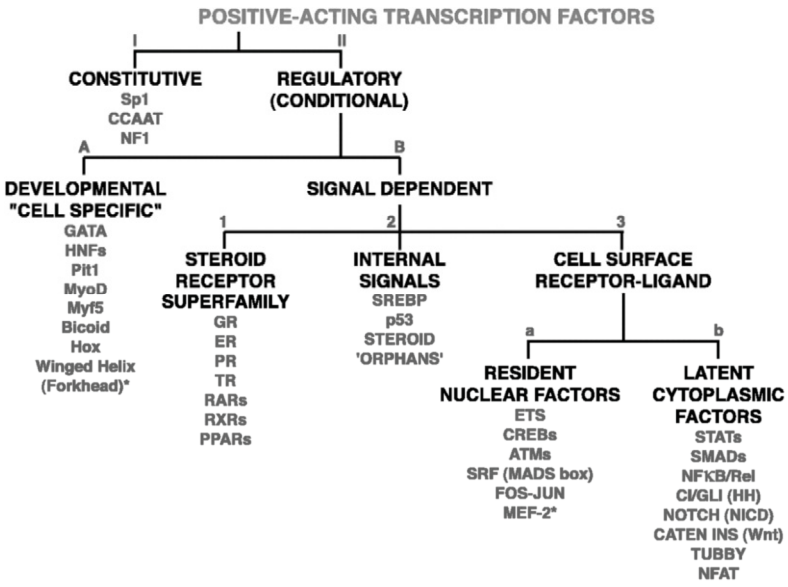

Figure 1 Functional classification of positive-acting transcription factors. Major functional groups are shown in black; specific examples are illustrated in grey. The list of examples is not complete, and can probably be applied on negative-acting transcription factors. An asterisk indicates that the indicated factors can be trapped in cytoplasm by phosphorylation. This figure is taken from [6]

activated upon ligand binding or post-translational modification. The last group can be further divided as shown in Fig 1. By categorizing the transcription factors in this way, one might get a better understanding of how the different factors are involved in signal transduction and how these pathways have evolved. However, this system also has its limitations, since all cell specific transcription factors by default are excluded from the signal dependent group. The signal-dependent transcription factors seem to be of particular interest in relation to carcinogenesis and thereby constitute potential therapeutic targets in cancer treatment. c-Myb which traditionally would have been placed in the group of developmental, cell specific factors, might actually belong to the class of signal-dependent factors, given the total body of knowledge on the significance of post-translational modification in regulating c-Myb activity. This will be further discussed in chapter 1.2.3.

When an mRNA-coding gene is to be expressed, the crucial regulatory event is the initiation of transcription. This requires assembly of the pre-initiation complex (PIC) onto the core promoter (Fig 2; reviewed in [11]). The PIC is composed of properly structured DNA, RNA polymerase II and the general transcription factors (TFIIB, TFIID, TFIIA, TFIIE, TFIIF and TFIIH). When all these factors have been gathered at the promoter, TFIIH which contains ATP-dependent helicase activity unwind the promoter DNA around the transcription start site and trigger the initiation of transcription. These actions and factors are common to the transcriptional initiation of expression of most regulated genes in the cell.

The specificity and level of gene expression are defined by combinatorial events upstream of the PIC assembly: When expressed and available, sequence-specific transcription factors (activators; Fig 2) can bind to their DNA recognition elements and recruit different co-factors. Co-factors often exert their function in larger complexes (co-activator or co-repressor complexes) which usually contain factors with histone modification and nucleosome remodelling activity. These activities modulate chromatin dynamics which today is recognized as a fundamental way to regulate gene expression [12-14]. By recruiting co-factor complexes DNA-binding transcription factors can poise a gene for transcription, or alternatively silence it. As shown in Fig 2, transcription factors bind to specific elements on the DNA that can be located in the proximity of the transcriptions start site (proximal promoter region) or to so-called distal enhancer elements. In both instances the transcription factors can be directly or indirectly involved in recruiting factors in the general transcriptional machinery, e.g. the TAFs (Fig 2). As stated above it is the balance between both activating and repressive events on the promoter that tunes the transcription of a certain gene. Therefore, mutations in or deregulation of transcription factors, or loss of

regulatory DNA-elements might be critical for the regulation of gene transcription, and eventually disastrous for the cell.

The importance of transcriptional regulation can be emphasized by an interesting observation: At the coding nucleotide level there is approximately 99% homology between man and chimpanzee. Both genomes encode proteomes that differ only at single amino-acid positions in homologues proteins. Still the difference between these two organisms is conspicuous. Thus, there are strong reasons to believe that the difference resides at the level of gene regulation [15], even though processes like alternative splicing and posttranslational modifications clearly contribute. An accurately regulated genome may therefore constitute the major difference between monkey and man, between nature and culture, but also between health and disease, benignancy or malignancy. In the following chapters I will focus on the transcriptional regulator c-Myb. Examples will be given of how the transactivational activity of c-Myb is modulated, and how it might be deregulated during oncogenesis.

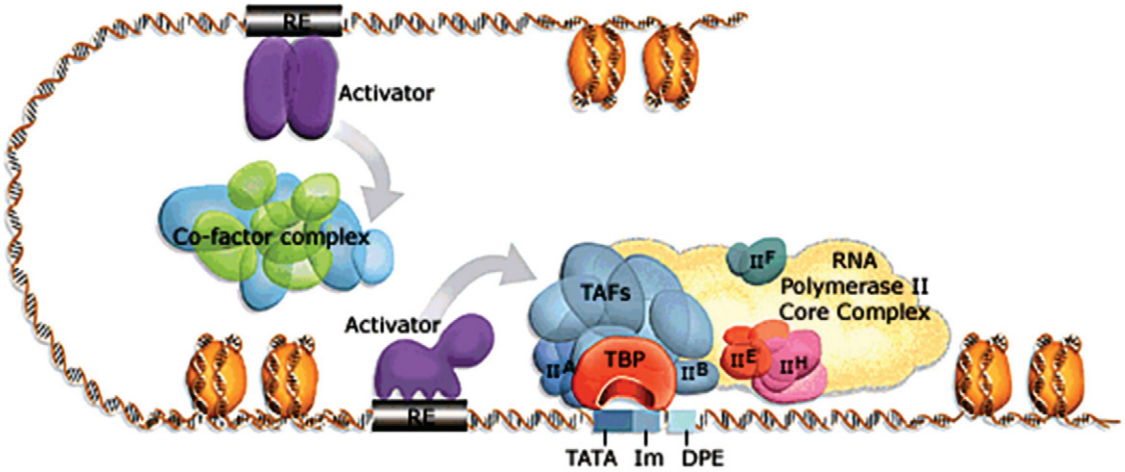

Figure 2 Initiation of transcription. The RNA polymerase II is directed by transcription factors, some of which exert their function in multisubunit complexes (co-factors). Some of these complexes can serve as bridges between sequence-specific transcription factors (activators) and the RNA polymerase machinery. One class of co-factors, called TAFs, join with TBP (TATA-binding protein) to form the TFIID complex, and attach to the TATA box at the gene's promoter. The combinatorial effect of the cells elaborate transcription apparatus regulates the rate of transcription. RE: Recognition Element. Illustration taken from the Robert Tjian lab's homepage (www.hhmi.org/research/investigators/tjian.html).

1.2 THE TRANSCRIPTION FACTOR c-MYB

The first member of the *myb* gene family identified was the v-*myb* oncogene from the avian ***my***elo***b***lastosis virus, AMV (reviewed in [16]). This acutely transforming retrovirus causes rapid and fatal monoblastic leukemia in chickens. Since the discovery of v-*myb* [17, 18] in

the 80's, and some years later the sequence of the cellular origin; c-*myb* [19], both gene products have been extensively studied. Still, many aspects of Myb-biology remain to be elucidated. This chapter will give a general overview of the c-Myb transcription factor, its structural relatives, functional domains, modifications, interaction partners and biological functions.

1.2.1 The Myb family of transcriptions factors

The structural characteristic of the members of the Myb family of transcription factors is the presence of one or more Myb domains, which make up the DNA-binding unit of these proteins. The Myb domain encompasses approximately 50 amino acids which contain a series of highly conserved residues with a characteristic spacing. This includes three regularly spaced Trp residues, some of which may be replaced by Phe, Ile or Tyr. Multiple copies of the Myb domain are frequently present as tandem repeats within a single protein (reviewed in [10]). The proto-oncoprotein c-Myb contains three such repeats, and in general, all Myb proteins found in animals contain three tandem repeats. In plants most Myb proteins contain two repeats, although proteins with only a single Myb domain have been found [20].

Three functionally distinct Myb proteins are known in vertebrates; A-Myb, B-Myb and c-Myb (Fig 3; [21]). c-Myb is the best characterized member in the family, and it is known to function as a transcriptional activator involved in regulating proliferation, differentiation and apoptosis in hematopoietic cells. In addition, c-Myb is also involved in the formation of colonic crypts through similar mechanisms. Like c-Myb, A-Myb functions as a transcriptional activator [22], being expressed at high levels in immature male germ cells, mammary gland ductal epithelium, a subset of B cells and in dividing neural precursor cells. In contrast to the restricted pattern of expression of A- and c-Myb, B-Myb is ubiquitously expressed and can be found in a variety of dividing cells. Here B-Myb functions as a transcriptional activator playing a general role in cellular proliferation (reviewed in [23]). All three vertebrate Myb proteins contain three functional domains (Fig 3): a DNA-binding domain (DBD with three Myb repeats (R_1, R_2 and R_3), a central acidic domain, which functions as a transactivation domain (TAD) in A- and c-Myb, and a negative regulatory domain (NRD). The DBD is the most conserved region among the Myb proteins. A- and c-Myb DBD share 90% identity within this region, while the homology between B- and c-Myb DBD is 75% [24, 25].

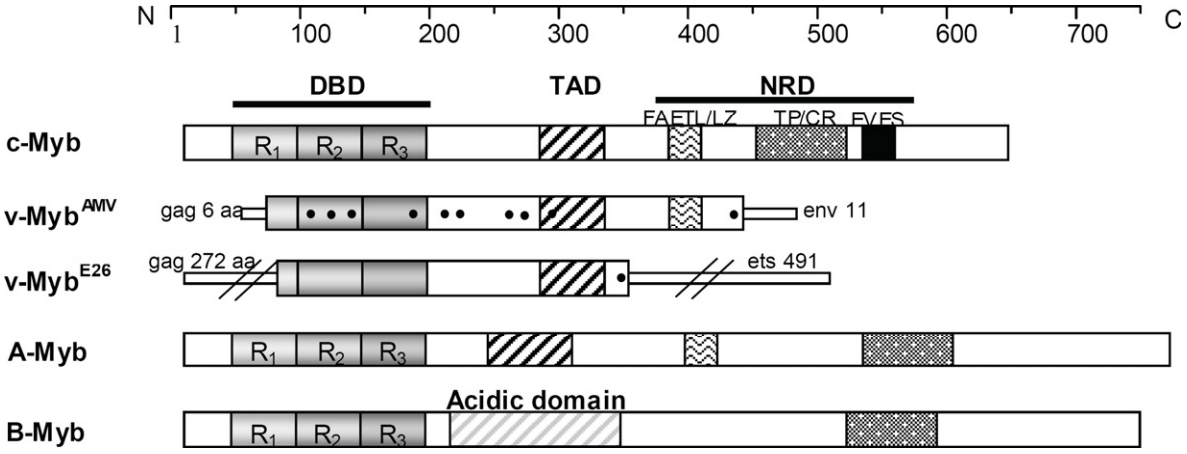

Figure 3 Comparison of the vertebrate Myb family proteins, including AMV and E26 v-Myb. Relative to c-Myb AMV v-Myb lacks 71 and 198 amino acid residues N- and C-terminally, respectively. In addition it carries 10 point mutations. v-*myb*^{AMV} also encodes 6 amino acids of the viral core protein Gag N-terminally, and 11 amino acids of the viral envelope protein Env C-terminally. E26 v-Myb is a Gag-Myb-Ets fusion protein, which lacks 80 and 277 amino acid residues relative to c-Myb N- and C-terminally, respectively. In addition it carries one amino acid substitution. The oncogenic mutations are shown as black dots. The DNA-binding domain (DBD) is highly conserved between A-, B and c-Myb. Both A- and c-Myb contains a central transactivation domain (TAD), whereas the central acidic domain in B-Myb does not confer transcriptional activity to the protein. The C-terminal of the three Myb proteins is less conserved, except for the Thr- and Pro-rich region (TP/CR). NRD: negative regulatory domain, LZ: leucine zipper, FAETL and EVES: motifs found within the assigned regions. $R_{1/2/3}$: Myb repeat 1, 2 and 3.

In addition to the three Myb-proteins expressed normally in vertebrates, two virus-transferred versions of c-Myb have been described, both in the chicken system; AVM and E26 v-Myb. AMV v-Myb, which was the first to be described [17, 18], contains a truncated and mutated form of chicken c-Myb. This was shown to be created by retroviral insertional mutagenesis, followed by recombination with the c-*myb* gene, and rescue by a replication competent helper virus [16]. AMV is an acutely transforming retrovirus which causes rapid and fatal monoblastic leukemia in chickens. E26, another retrovirus which causes an acute erythroblastic leukemia in chicken and transform multi-potent hematopoietic precursor cells in culture [26, 27] was found to contain another truncated version of c-Myb, termed E26 v-Myb. Both viral Myb proteins have N- and C-terminal deletions relative to the cellular homologue c-Myb (Fig 3). In addition AMV v-Myb harbours 10 amino acid substituting mutations along with N- and C-terminally fused remnants of the viral Gag and Env proteins. E26 v-Myb on the other hand is part of a larger fusion protein with 272 amino acids of the viral gag protein fused N-terminally and 491 amino acids of the cellular protein Ets-1 fused C-terminally (Fig 3; extensively reviewed in [16]).

1.2.2 The structural and functional domains of c-Myb

Like most other transcription factors, the overall structure of c-Myb is modular, with functional domains that can act as separate units. The c-Myb protein harbours three such functional domains: the N-terminal DNA-binding domain (DBD), a central transcriptional activation domain (TAD) and a C-terminal negative regulatory domain (NRD). In this chapter the main features of these domains will be highlighted.

1.2.2.1 The DNA-binding domain – DBD

The c-Myb DBD consists of three conserved and closely related Myb domains, designated R_1, R_2 and R_3 (Fig 4). Each repeat contains three regularly spaced tryptophan residues that are important for maintaining an active DNA-binding structure [28, 29]. In addition these domains have conserved acidic and hydrophobic regions and single position residues (Gly, Arg and Leu; Fig 4).

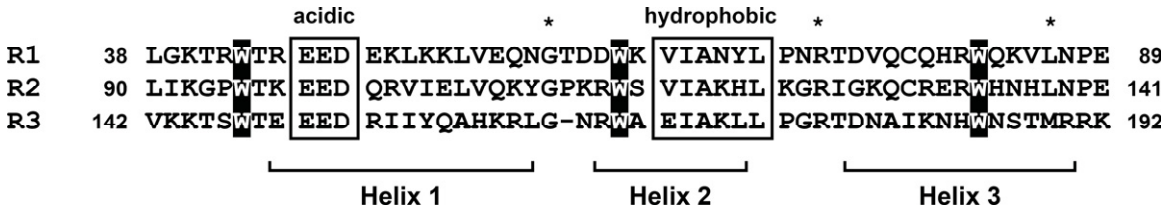

Figure 4 Multiple sequence alignment of the three Myb domains in c-Myb DBD. R1, R2 and R3 repeat of human c-Myb aligned by the conserved Trp residues (black shading). Conserved acidic and hydrophobic residues/stretches are boxed. Conserved single residues are marked with an asterisk. The secondary structure elements (α-helical regions) are indicated by brackets.

In 1991 Gabrielsen and co-workers presented data suggesting that the R_2R_3 part of DBD built up a double helix-turn-helix (HTH) related motif [30]. This provided the first experimental evidence for a hypothesis put forth some years earlier [31]. Secondary structure predictions and mutational analyses demonstrated that the HTH motifs in both R_2 and R_3 contributed to sequence-specific DNA-binding [30]. Shortly thereafter Frampton *et al*. reported a similar study [32]. The common concept was that each c-Myb repeat consisted of three α-helices, maintained by a hydrophobic core built around the conserved tryptophans. Finally, NMR studies of $R_1R_2R_3$, R_2R_3 and single repeats confirmed the proposed structure ([33-36]; Fig 5), even though some disagreement arose concerning the organization of the second repeat in solution [36, 37]. It is now thought that a distorted region in the R_2 C-terminal, corresponding to its DNA recognition helix, becomes structured and completes the HTH-related motif upon binding of DNA [33, 37, 38].

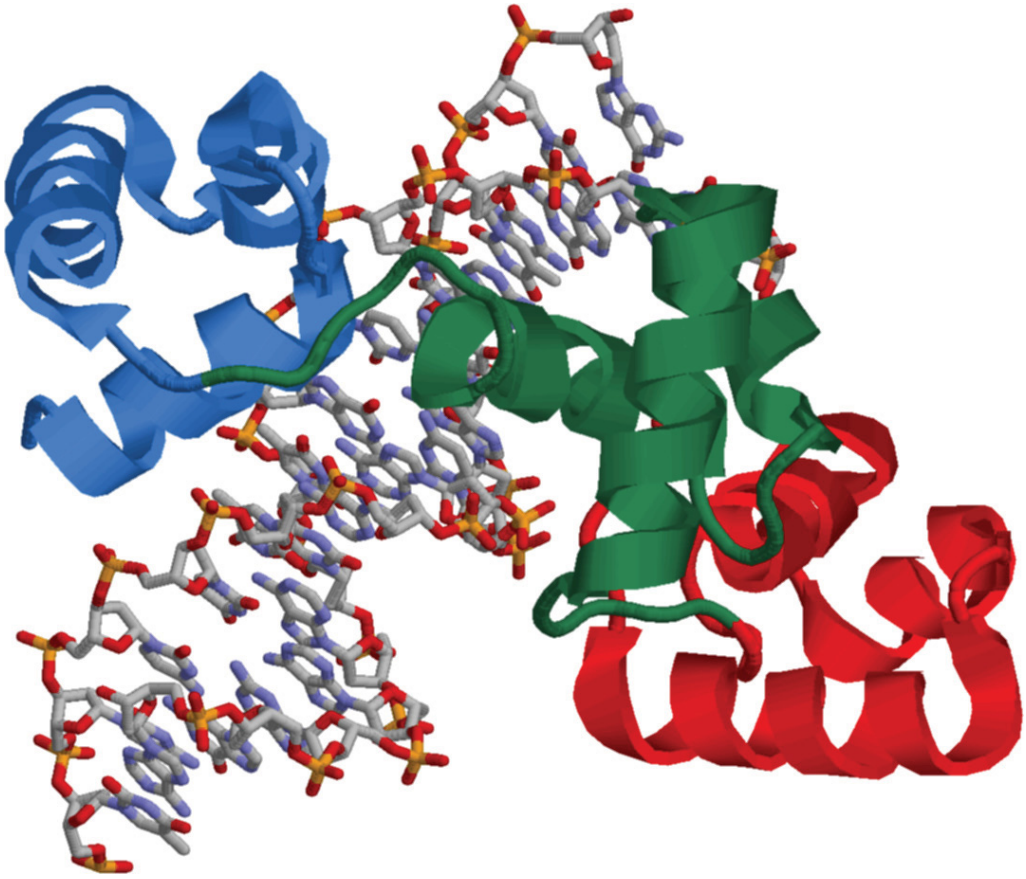

Figure 5 Three-dimensional structure of c-Myb $R_1R_2R_3$ in complex with DNA. Myb repeats R1, R2 and R3 are shown in red, green and blue, respectively. The recognition helices of R2 and R3 are oriented along the major groove, while R1 has a more peripheral localization in the complex. The picture was generated from the crystal structure data of Tahirov *et al.* [39] using RasWin v2.7.2.1.1.

The c-Myb protein binds to DNA as a monomer [40, 41], and deletion studies have shown that the R_2 and R_3 repeats are required and sufficient for DNA-binding [40, 42]. The dispensability of R_1 is demonstrated by AMV and E26 v-Myb. Both proteins have lost this repeat, but are still capable of binding DNA and activate transcription (Fig 3). Further NMR studies and crystal structure of the c-Myb:DNA complex revealed that it is the third helix in R_2 and R_3 that recognize DNA [33-36, 39]. These two recognition helices contact the major grove of DNA in a head-to-tail fashion (Fig 5; [35]). Furthermore, specific amino acids in the linker between the two repeats seem to play an important role for sequence-specific DNA-binding [43]. The recognition sequence of c-Myb (Myb recognition element; MRE) was initially determined to be YAACKG by Biedenkapp and co-workers [44] and later extended to YAACBGYCR and YAACKGHH by two binding site selection studies [45, 46]. Based on the fact that two of the Myb repeats bind DNA, the MRE can be divided into half-sites. The folded R_3 domain is involved in the recognition of the first half-site; YAAC, while the more flexible R_2 contacts the second half-site. More recent investigations have

added further constraints to the binding site requirements, especially the preference for a T-rich stretch in the second half-site [47, 48].

In 1996 Aasland and co-workers [49] suggested that the Myb repeats might be related to, and give insight to the function of, a novel protein motif termed the SANT-domain. This ~50 amino acid long protein motif was found in several chromatin regulatory proteins including Swi3, Ada2, N-CoR and TFIIIB (hence the name). The Myb repeats, including the aromatic Trp signature residues, aligned surprisingly well with other SANT-domains [49]. However, while the Myb-repeats bind DNA, the SANT-domains have been shown to bind to histone tails [50, 51]. Despite the lack of enzymatic activity, the SANT-domains are functionally involved in histone acetylation, deacetylation, and ATP-dependent remodelling [50, 52]. This might be facilitated through the bipartite function of SANT, serving as a protein interaction domain recruiting histone modifying enzymes, while simultaneously facilitating substrate recognition (reviewed in [53]).

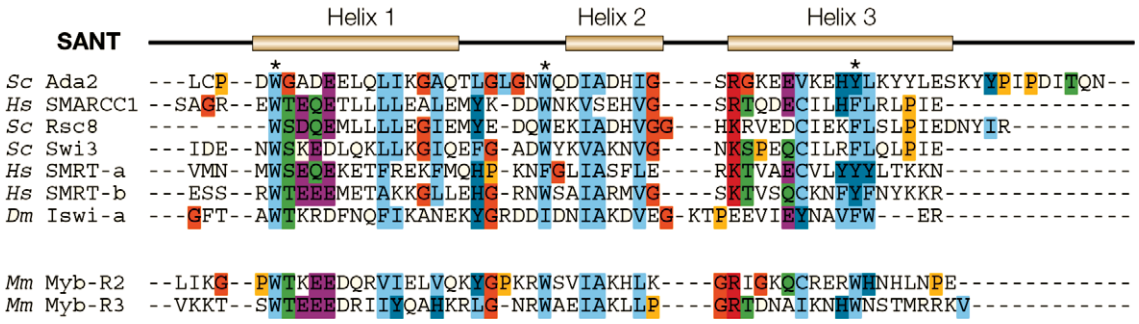

Figure 6 The SANT domain: a highly conserved motif that is similar to Myb DBD. Multiple sequence alignment of SANT- (Swi3, Ada2, N-CoR and TFIIIB) containing sequences. Columns of residues that show sequence similarity are coloured according to their respective properties. The secondary structures shown above the alignment correspond to the Iswi crystal structures. Asterisks highlight the bulky residues that are predicted to form the hydrophobic core of the SANT domain. *Dm*: *Drosophila melanogaster*, *Hs*: *Homo sapiens*, *Mm*: *Mus musculus*, *Sc*: *Saccharomyces cerevisiae*, Ada2: adaptor 2, Rsc: remodel the structure of chromatin, SMARCC1: SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, SMRT: silencing mediator of retinoid and thyroid receptors. Adapted from [53].

For several years the function of the c-Myb SANT-domain remained elusive, and a direct connection between Myb domains and chromatin modification was yet to be shown. Then in 2005, Mo and co-workers demonstrated that c-Myb DBD also binds to the N-terminal tail of histone H3 between amino acid residues 27-42 and facilitates acetylation of H3 lysines [54]. Furthermore, they could show that the leukemogenic mutations in AMV v-Myb eliminated this interaction and consequently acetylation of H3 tails. This revealed the mechanism behind the lack of v-Myb induced *mim-1* (Myb-induced myeloid protein 1) gene expression (see chapter 1.2.4.2).

1.2.2.2 The transactivation domain – TAD

The transactivation domain of c-Myb is localised near the centre of the protein (Fig 3). Despite the early recognition, it has been difficult to define its exact localisation. The c-Myb TAD was first identified in chicken c- and v-Myb [42, 55]. Both proteins can activate transcription on synthetic reporter constructs that contain multiple MREs [42, 55-57]. The initial mapping was done with various deletion constructs of c-Myb or v-Myb proteins fused to a heterologous DNA-binding domain (GAL4p). These mappings defined the TAD to be positioned between amino acid 275 and 325 in mouse and chicken c-Myb ([42, 55, 58]; same positions for human c-Myb; Fig 7). This region is the minimal fragment sufficient to activate gene expression when fused to Gal4 DBD. Nevertheless, transactivation by c-Myb requires both a functional DBD and TAD. The c-Myb TAD resembles other described transactivation domain in that is hydrophilic and slightly acidic (Fig 7). However, none of the acidic residues seem to be essential for transcriptional activation [59], rather several redundant regions seem to cooperate to achieve full transactivating ability in the full-length protein [59, 60].

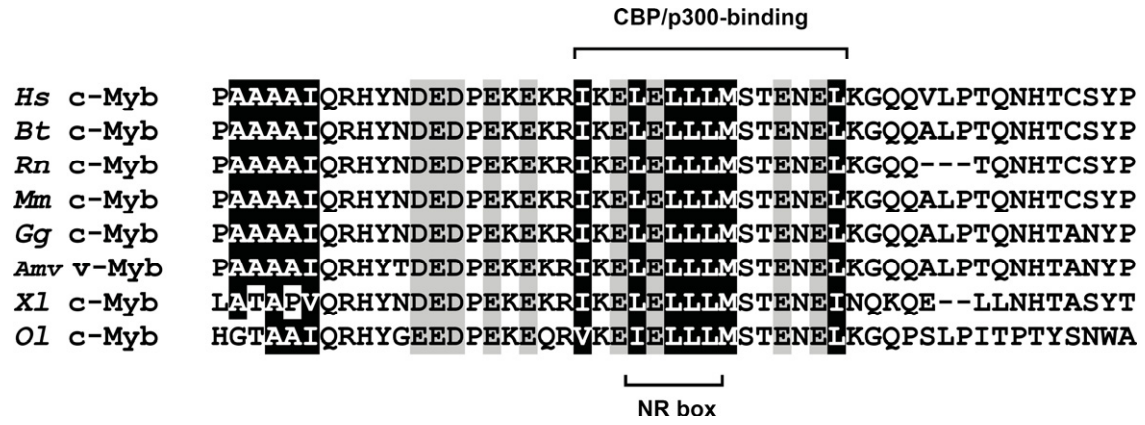

Figure 7 The minimal transactivation domain of c-Myb. Multiple sequence alignment of TADs from vertebrate c-Myb and AMV v-Myb, corresponding to amino acid residues 275-325 in human c-Myb. Hydrophobic residues are shaded in black, while acidic residues are shaded in grey. The area interacting with CPB/p300 is indicated with brackets above the MSA, while the Nuclear Receptor (NR) box is specified below. *Hs: Homo sapiens, Bt: Bos Taurus, Mm: Mus musculus, Rn: Rattus norvegicus, Gg: Gallus gallus, Xl: Xenopus laevis Ol: Oryzias latipes.*

The TAD in c-Myb is able to directly recruit CBP (CREB-binding protein) and p300 (see chapter 1.2.3.1). These closely related histone acetyl transferases (HATs) bind via their KIX domain to amino acids 295-309 in TAD (Figs 7 and 8). A general effect of HATs is the acetylation of histone lysines and consecutive de-condensation of nucleosome structure, compatible with gene activation. Within the CBP/p300 binding sequence of c-Myb TAD

there is a small LXXLL-motif called the nuclear receptor (NR) box ([61, 62]; Fig 7), which appears commonly in complexes formed between NRs and their co-activators. Interestingly, structures of the complexes between NRs and co-activators from the p160 family [63-65] resemble the c-Myb:KIX complex, where an amphipathic helix containing the LXXLL sequence is bound in a hydrophobic groove (Fig 8; [66]). Some studies indicate that the amphipathic helix in the CBP/p300-interacting region of c-Myb TAD stabilise upon binding [67, 68]. Moreover, structural and isothermal titration calorimetry data show that in contrast to other CBP/p300-interacting proteins, c-Myb does not need to be phosphorylated in the interacting region to facilitate binding [66, 67].

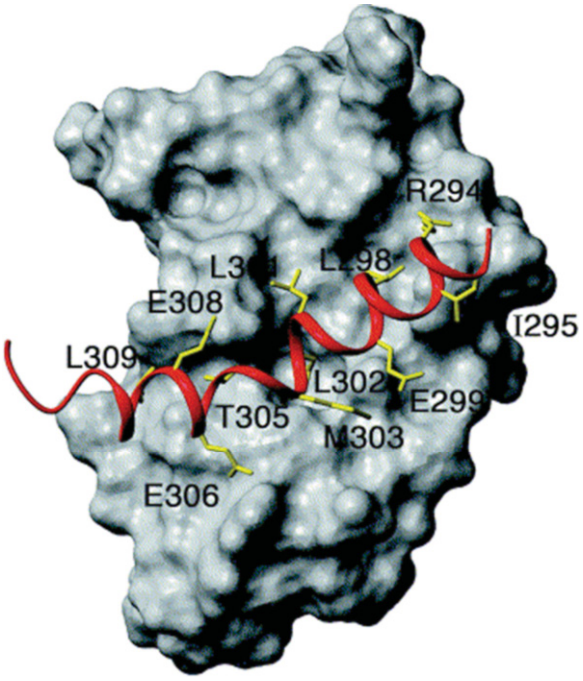

Figure 8 The complex between CBP KIX and c-Myb. The CBP KIX is shown as a grey surface, while the backbone of the amphipathic helix in c-Myb TAD is shown as a red ribbon. The side-chains of c-Myb that interact with CBP KIX are shown in yellow. Taken from [66].

1.2.2.3 The negative regulatory domain – NRD

The C-terminus of c-Myb has been termed the negative regulatory domain (NRD). However, the exact boundaries of the domain have been difficult to define due to conflicting evidence. One of the reasons for this is that the function of some of the NRD subdomains is still unclear. The initial idea of a carboxy-terminal NRD came from observations of the v-Myb protein which lacks the C-terminus, while at the same time is a more potent activator than c-Myb. Deletion of the c-Myb C-terminus, like in the viral forms, dramatically increases the transactivation potential of the protein [42, 69]. The NRD encompasses three subdomains; FAETL, TP/CR and EVES (Fig 3). It is generally accepted that the threonine- and proline-rich region (termed TP/CR) and the domain containing the EVES-motif (termed EVES) could be viewed as negative determinants of c-Myb activity. Whether or not the

FAETL-domain, a leucine-rich region positioned N-terminal of TP/CR, should be included has been a matter of debate (reviewed in [8]).

The leucine-rich **FAETL domain** is by many termed the leucine zipper region (LZ), due to the presence of a heptad leucine repeat (HLR) within this domain (Fig 9). It has been predicted to form a coiled-coil α-helix structure when interacting with other HLR-containing proteins [70]. Interestingly the HLR-containing protein p160 has been demonstrated to bind to this region of c-Myb [71, 72]. However, Ebneth and co-workers were unable to detect any α-helical structure of the putative LZ domain in chicken c-Myb [73]. The LZ-region has been shown to promote the formation Myb-Myb dimers *in vitro*, which are unable to bind DNA [74]. However, these findings have been questioned by others [75]. Within the leucine-rich region there is a stretch of 10 amino acid residues which include the FAETL-motif that has been shown by mutational analyses to be essential for both transcriptional activation and oncogenic transformation by v-Myb [56, 60]. It should be mentioned, however, that E26 v-Myb, as opposed to AMV v-Myb, has disposed itself of this region (Fig 3).

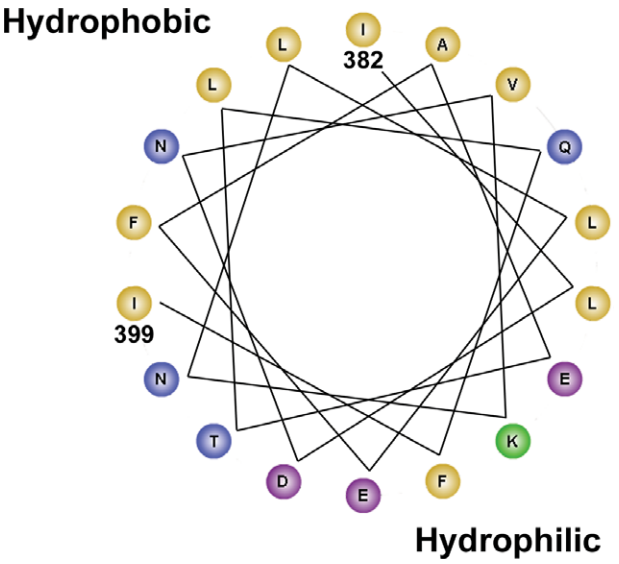

Figure 9 The heptad leucine repeat in c-Myb might make a leucine zipper. The heptad leucine region of c-Myb NRD represented as a helical wheel (I382-I399; human c-Myb numbering). Hydrophobic residues in beige, acidic, hydrophilic residues in purple and basic in green, polar residues in blue. Illustration made with 'Helical wheel' made by John K. Everett, CABM Structural Bioinformatics Laboratory (www-nmr.cabm.rutgers.edu).

The **TP/CR domain** is the most conserved region of c-Myb outside the DBD and contains the sequence TPTPFK, found in all vertebrate A-, B-, and c-Myb proteins. This motif has been implicated in negative regulation of c-Myb activity [76, 77]. Nevertheless, several lysine residues around this sequence (K442, K445, K471, K480 and K485; human numbering) have been shown to be acetylated, resulting in an increase in c-Myb transactivational activity [78, 79]. The TP/CR domain also contains seven conserved

potential phosphorylation sites for proline directed kinases. Some of these sites are probably involved in regulating the stability of c-Myb [80].

The most C-terminal of the three NRD sub domains is the **EVES domain**. The EVES motif, which has given the domain its name is only found in vertebrate c-Myb proteins, but is well conserved within this phylum. Our group and others have reported that Lys503 and Lys527 (human numbering) in the NRD can be covalently modified by the Small Ubiquitin-related Modifier, SUMO ([81-83]; see chapter 1.2.3.2). Both SUMO-1 and SUMO-2/3 modification of c-Myb result in lowered transcriptional activity [81-83]. The EVES domain also contains a site for proline-directed phosphorylation. Ser532 (human numbering) has been reported to be phosphorylated by $p42^{mapk}$ [84-86] which lowers the c-Myb activity in a degradation-independent manner [87, 88]

The mechanism behind this negative regulation by the NRD is probably multifaceted. However, three lines of evidence should be mentioned; recruitment of co-repressors (I), posttranslational modifications (II) and intra-molecular interactions (III). **I:** Based on the current list of factors reported to interact with c-Myb (see chapter 1.2.3.1 and Table 1) there is an overrepresentation of negatively acting factors among the ones binding to NRD, e.g. p67/p160 [71, 72, 89] TIF1β (co-recruiting mSIN3A, N-CoR and c-Ski; [90]), and BS69 [91]. **II:** Several sites of negatively acting post-translational modifications have been reported. Sumoylation of Lys503 and Lys527 result in lowered transcriptional activity of c-Myb [81-83], while phosphorylation of several SP and TP sites lead to lowered activity in a degradation-dependent [80] and -independent manner [87, 88]. **III:** Some experiments have suggested that the C-terminal part of c-Myb can inhibit DNA-binding [92]. This reduction in DNA-avidity has been proposed to be caused by intra-molecular interactions in c-Myb. Specifically, the N- and C-terminus (DBD and EVES) were shown to score positively for protein-protein interaction in yeast two-hybrid and phage display assays [93, 94]. However, other investigators have failed to observe this inhibition of DNA-binding by the NRD [75], and the data supporting the N- to C-terminal intermolecular contact have been difficult to reproduce (T.Ø. Andersen, unpublished results).

1.2.3 Regulation of c-Myb activity

Apart from regulating the level of c-Myb through modulating transcription of the c-*myb* gene, the cell has two main processes to implement changes in c-Myb activity: 1) interaction with other proteins and 2) posttranslational modifications. These processes are, as for most other proteins, interlinked. However, they will be treated in separate chapters for clarity.

1.2.3.1 c-Myb interacting proteins

Through the interaction with other nuclear proteins the sequence-specific transcription factors can increase their affinity and specificity for DNA, they can tune their transactivational activity, remodel chromatin, modify histones and/or in some instances contact the general transcriptional machinery. It has, however, become evident that transcription factors typically exert their function in larger protein complexes, which make the study of such co-factors challenging. This is also the case for c-Myb. Several proteins have been reported to interact with different subdomains of c-Myb and influence its activity (Table 1; reviewed in [95]). Still, complete multimeric complexes have so far not been described in greater detail, and establishing a catalogue of well documented, direct interaction partners of c-Myb is so far a task unfinished. In this chapter two of the best described co-operating factors of c-Myb; C/EBPβ and CBP/p300 will be highlighted. In addition recent data on c-Maf and MafB are included, through which a reciprocal MafB-c-Myb repression is linked to sumoylation.

The transcription factor **C/EBPβ** (CAAT/Enhancer Binding Protein) directly interacts with, and cooperates with c-Myb in activating myeloid-specific genes [39, 96, 97]. The crystal structure of the ternary complex with c-Myb DBD, C/EBPβ and DNA from the *tom-1* promoter (see chapter 1.2.4.2) showed that the R_2 repeat of c-Myb DBD, bound to DNA, interacted with the C-terminal part of the DNA-bound C/EBPβ DBD. Moreover, since C/EBPβ and c-Myb bind at a distance to the *tom-1* promoter, atomic force microscopy demonstrated that this interaction was possible due to looping of DNA [39]. It should however be mentioned that looping of DNA is not required on all C/EBPβ and c-Myb-responsive promoters. At the *mim-1* promoter the factors bind to two adjacent sites and efficiently induce gene expression [96, 97].

One of the best described interactions of c-Myb is the association with the acetyl transferase and transcriptional co-activator **CREB-binding protein** (**CBP**) and its close homologue; **p300** [98, 99]. CBP/p300 has been shown to bind to a short motif in c-Myb TAD via its KIX domain (see chapter 1.2.2.2 and Fig 8). An additional interaction surface is found between the leucine-rich FAETL domain of c-Myb and the C/H2 domain of CBP/p300 [78]. The c-Myb transactivation potential is stimulated by CBP/p300, indicating that c-Myb is a target for the co-activator function of this protein. This finding was confirmed by the inhibition of Myb-dependent gene activation when lowering the expression of CBP by antisense mRNA or dominant negative mutants [98, 99]. Over the last

Protein	Myb dom.	Evidence	References
C/EBPβ	DBD	funct.dep, CoIP+/+, GST, X-ray	[39, 100]
Cyclin D1/D2	DBD	end-CoIP+/-, Y2H, funct. dep.	[101]
c-Myb EVES	DBD	Y2H, yeast CoIP+/-	[93]
p100	DBD	Y2H, *in vitro* CoIP+/-, funct. dep.	[93]
Cyp40	DBD	GST, funct. dep.	[102]
Nucleolin	DBD	GST, end-CoIP+/-, funct. dep.	[103]
c-Maf	DBD	end-CoIP+/-, GST, Y2H, EMSA, funct.dep.	[104, 105]
MafB	DBD	GST, funct. dep.	[106]
HSF3	DBD	EMSA, GST, Y2H, funct.dep.	[107]
Pim-1	DBD		[108]
c-Ski	DBD	Y2H-scr, CoIP+/-, GST, funct. dep.	[90]
N-CoR	DBD	Y2H-scr, CoIP+/+, GST, funct. dep.	[90]
mSin3A	DBD	Y2H-scr, CoIP+/-, GST, funct. dep.	[90]
HIPK2	DBD	end-CoIP+/-, GST, funct. dep.	[109]
NLK	DBD	end-CoIP+/-, GST, funct. dep.	[109]
TRAF7	DBD	end-CoIP+/-, GST, funct. dep.	[110]
RARα	DBD+NRD	GST, Y2H	[111]
CBP/p300	TAD+NRD	Y2H, funct. dep, GST, NMR, X-ray	[66, 67, 78, 98, 99]
ATBF1	TAD	Y2H-scr, GST, CoIP+/-, funct. dep.	[112]
p160/Mybbp1a	HLR	Pull-down, funct.dep.	[71, 72, 89]
BS69	NRD	Y2H-scr, *in vitro* CoIP, funct. dep.	[91]
TIF1β	NRD	Y2H-scr, CoIP+/-, GST, funct. dep.	[90]
Rcd-1	NRD	Y2H-scr, GST, funct. dep.	[113]
Ubc9	NRD	Y2H-scr, funct. dep.	[81, 82]
HES-1	Unknown	end-CoIP +/-, funct. dep.	[114]
Pax-5	Unknown	CoIP+/+, funct. dep.	[115, 116]
PML	Unknown	end-CoIP+/-, GST, funct. dep.	[117]
LEF-1	Unknown	CoIP+/+, funct. dep.	[116]

Table 1 Proteins reported to interact with c-Myb. The candidates are sorted according to the c-Myb domain involved. Y2H: two-hybrid system, Y2H-scr: Yeast two-hybrid screening, GST: GST pull-down assay, CoIP: co-immunoprecipitation *in vitro* or in cell extracts from transfected cell lines, end-CoIP: co-immunoprecipitation of endogenously expressed proteins, +/+: CoIP successful in both directions, +/-: only one-way CoIP reported/successful, funct. dep.: functional dependence, EMSA: super shift in EMSA (electrophoretic mobility shift assay), X-ray: X-ray diffraction, NMR: nuclear magnetic resonance.

years several sequence-specific transcription factors have been shown to interact with CBP/p300. Some of the CBP/p300-interacting transcription factors are activated directly by acetylation, some rely on CBP/p300s ability to acetylate histone tails and thereby activating transcription, while others exploit both mechanisms. c-Myb seems to fall in the last category. Studies show that CBP/p300 can acetylate several Lys residues in the conserved TP domain of c-Myb, with different lysines as substrate (p300: K471, K480 and K485; CBP: K442, K445, K471, K480 and K485) [78, 79]. Acetylation of c-Myb by CBP was suggested to increase CBP's affinity for c-Myb, thereby increasing the co-activation [78]. The fact that c-Myb DBD binds to the N-terminal tail of histone H3, facilitates acetylation of H3 lysines

and induce gene expression [54], is an example of how c-Myb utilizes the second CBP/p300 function, namely the histone acetyl transferase activity.

c-Maf is an example of a protein that interacts with c-Myb to reduce its transcriptional activity. Expression of c-Maf in human immature myeloblastic cells inhibited c-Myb induced *CD13/APN*-driven reporter gene activity through the binding to c-Myb DBD [104]. This effect was dependent of the developmental stage of the cells, since formation of inhibitory Myb-Maf complexes changed through differentiation, with the levels being highest in immature myeloid cells [104]. Recently, c-Maf was also shown to downregulate *bcl-2* expression in TCR-engaged $CD4^+$ cells in a c-Myb dependent manner. This was suggested to be caused by c-Maf-c-Myb interaction disrupting the binding of c-Myb to the promoter [105]. Interestingly, the transcription factor **MafB,** a closely related Maf-family member also binding to c-Myb DBD, was recently shown to reciprocally inhibit transactivation together with c-Myb [106]. This reciprocal inhibition by the two factors was demonstrated to be highly dependent on sumoylation of both MafB and c-Myb (see chapter 1.2.3.2 and 1.3). Since MafB promotes the differentiation of myeloid progenitors towards the macrophage lineage, while c-Myb can maintain proliferation and block differentiation, these findings suggest that SUMO modification of MafB and/or c-Myb can affect the balance between myeloid progenitor expansion and terminal macrophage differentiation [106].

As evident from the list in Table 1, more proteins have been reported to interact with c-Myb. However, both the molecular and physiological relevance of several of the reported interactions remain to be determined. To extend the knowledge of proteins interacting with c-Myb, we previously carried out a yeast two-hybrid screening in a mixed cDNA library (human bone marrow and human erythroleukemia cell line K562) using full-length c-Myb as bait [81]. Two of the interacting proteins picked up in this screening; the chromatin remodelling factor **Mi-2α** and the huge nuclear factor **FLASH**, reported to be essential for cell division, have been studied further in our group. The work on these two factors are included in this thesis (see PAPER I and II). Another interacting protein detected in our screening; the SUMO E2 conjugating enzyme **Ubc9** (see chapter 1.3.2.2), which linked c-Myb to **SUMO**, was reported by our group some time ago [81]. Finding that c-Myb activity was regulated by sumoylation, led to the initiation of the two last studies reported in this thesis (see PAPER III and IV).

1.2.3.2 Post-translational modification of c-Myb

The other main mechanism for tuning the activity of the c-Myb protein is posttranslational modifications, and four types of modification have been described; phosphorylation, acetylation, ubiquitination and sumoylation. For covalent modification of a protein to occur an enzymatic activity is almost always required. Thus, c-Myb is dependent on interacting with nuclear enzymes for these modifications to take place. Furthermore, posttranslational modifications of transcription factors often do not change their activity *per se*, but allow for, or exclude, interactions with other co-factors. This is also believed to be the case for c-Myb. Altogether, it is evident that posttranslational modifications and the repertoire of interacting proteins are interdependent.

Phosphorylation

The N-terminus of c-Myb has been showed to be phosphorylated by casein kinase II (CKII) both *in vitro* and *in vivo* on amino acid residues Ser11 and Ser12 [118], however, some disagreement exist about whether this phosphorylation reduces the DNA-binding and cooperativity with NF-M (C/EBPβ) or not [118-121]. Studies in our lab have shown that Ser116 in R_2 in DBD c-Myb is phosphorylated by protein kinase A (PKA), while the corresponding residue in AMV v-Myb is not. This is due to mutation of the neighbouring residue, V117D (c-Myb numbering), in v-Myb [122]. Phosphorylation of Ser116 destabilizes the DBD-DNA complex *in vitro* and prevents c-Myb-dependent activation of chromosomal target genes [122]. The C-terminus of c-Myb contains a number of phosphorylation sites for proline-directed kinases and MAP kinases [84-86]. Hyper-phosphorylation of NRD is associated with targeted degradation of c-Myb (see chapter 1.2.2.3) [88, 123]. This is probably caused by phosphorylation-dependent ubiquitination events (see Ubiquitination).

Acetylation

c-Myb can be acetylated on five lysine residues in the conserved TP domain (K442, K445, K471, K480 and K485) by CBP and p300 (see chapter 1.2.3.1; [78, 79]). Acetylation of c-Myb by CBP has been proposed to enhance the interaction between CBP and c-Myb and thereby increasing the co-activation by CBP [78]. Lysine to arginine mutations in these five sites results in a marked reduction in the transactivation potential of c-Myb. Furthermore, the study of Sano and Ishii indicated that acetylation of all the five seats synergistically enhances c-Myb activity [78].

Ubiquitination

The NRD has been shown to be covalently modified by polyubiquitin chains on one or more unidentified lysine residues, which leads to recognition and rapid degradation by the 26S proteasome [124, 125]. When deleting the C-terminal part of c-Myb, the protein becomes more stable. Determinants for degradation, or degrons, have been identified within the leucine-rich region and in the very C-terminus (last 87 residues; [126]). Furthermore, deleting amino acid residues 358-452 (covering the FAETL domain and part of the conserved TP region) makes human c-Myb degradation-resistant, and enhances its proliferative potential in hematopoietic cells [127]. These sequences may be involved in some aspect of the ubiquitin modification.

Sumoylation

Another ubiquitin-related protein that participates in the regulation of c-Myb through covalent modification is SUMO, or Small Ubiquitin-related MOdifier (see chapter 1.3). Bies and co-workers have shown that murine c-Myb is conjugated to SUMO-1 at K499 and K523 [82], while Dahle and co-workers demonstrated sumoylation of human c-Myb at K503 and K527 (corresponding residues), using different methods [81]. Recently it was shown that c-Myb also can be SUMO-2/3-modified at K503 and K527 ([83]; human c-Myb numbering). When c-Myb is sumoylated by either SUMO-1 or SUMO-2/3 the transactivational activity is reduced. Conversely, mutating the SUMO acceptor lysines causes a superactive phenotype [81, 83]. Through the work on SUMO-1 modification both groups identified the K527 as the principal sumoylation site, being much more efficiently sumoylated than K503, and having a much more pronounced effect on c-Myb activity [81, 82]. Sumoylation of c-Myb is also proposed to increase the proteolytic stability of the protein [82]. This is not due to competition between SUMO and ubiquitin for acceptor lysines, but thought to be caused by some kind of shielding of ubiquitination/degradation related epitopes.

Modification by SUMO-2/3 has been demonstrated to be induced by cellular stress, but independently of the stress-activated protein kinases of the p38/MAPK and JNK families [83]. Heat stress, osmotic stress and metabolic stress, but not genotoxic stress increases conjugation of SUMO-2/3. While the SUMO E3 ligase PIASy seem to be most important for SUMO-1 conjugation to c-Myb [81], PIAS3 seem to be the major ligase involved in SUMO-2/3 modification [83].

Finally, speculative data have been presented in a report indicating that TRAF7 can function as a SUMO E3 ligase for c-Myb, sequester sumoylated c-Myb in the cytoplasm, and thereby lowering c-Myb transactivational potential [110]. However, unpublished data from our group using c-Myb-SUMO-1 fusion constructs to mimic constitutively sumoylated Myb, indicate that c-Myb still translocates to the nucleus (AH. Alm-Kristiansen unpublished data). This makes the data on cytoplasmic retention, difficult to comprehend.

1.2.4 The biology of c-Myb

c-Myb has traditionally been described as a hematopoietic transcription factor which is expressed in progenitor cells in the erythroid, lymphoid and myeloid lineages [128, 129]. The expression of c-*myb* is then downregulated during terminal differentiation (Fig 10; [130, 131]). The hematopoietic system has for many years been the biological system in which c-Myb function has been studied, and it is now generally accepted that c-Myb contributes to the regulation of proliferation, differentiation and apoptosis in hematopoietic cells [132, 133]. There is, however, a growing list of other tissues in which c-*myb* is expressed, e.g. neural retina and lung epithelium [129], aortic smooth muscle cells [134], the thyroid gland and hair follicles [135], neural progenitor cell [136] and colonic crypts [137]. Still, I have chosen to focus on the hematopoietic system in the following chapters.

1.2.4.1 The hematopoiesis

The high level of c-Myb in hematopoietic progenitors clearly indicate a function in the development of blood cells, and c-Myb was early suggested to play a role in the choice between proliferation and differentiation during blood cell formation. Expression of c-*myb* is associated with proliferation and the maintenance of an immature phenotype of hematopoietic cells. The first clue to this came from the observation that the oncogenic v-*myb* transforms hematopoietic cells with an immature phenotype [138, 139]. Furthermore, ectopic expression of c-*myb* was shown to block induced differentiation of several hematopoietic cell lines [140]. In contrast to the immature proliferating progenitor cells, mature non-proliferating hematopoietic cells do not express c-*myb* ([130, 141-143]; Fig 10).

At the molecular level c-Myb has been suggested to regulate genes associated with cell growth, including c-*myc* [144-148] , DNA topoisomerase IIα (*TOPIIα*; [149]) fibroblast growth factor 2 (*FGF-2*; [150]) and c-*kit* [151, 152]. Furthermore, c-Myb has been implicated as being anti-apoptotic through the activation of *bcl-2* expression [148, 153-155]. Over-expression of c-*myb* has also been shown to protect CTLL-2 cells from apoptosis

induced by interleukin-depletion [156]. Still, opposite effects have been seen for other cell lines [157-159], it therefore remains unclear whether the primary role of c-Myb is to prevent apoptosis or simply keeping cells in a cycling mode (reviewed in [8]).

In 1991 Mucenski and co-workers reported the homozygous c-*myb* null mouse, and showed that it develops normally up to embryonic day 13, but die on day 15 due to failure in the foetal liver hematopoiesis [160]. A more direct evidence for the role of c-Myb in hematopoietic cell proliferation comes from a series of experiments done with c-*myb* specific antisense oligonucleotides. The inhibition of c-*myb* expression by these oligonucleotides significantly reduced the proliferation of bone marrow and peripheral blood mononuclear cells, human myeloid cell lines and T lymphocytes (reviewed in [8, 132, 161]. Some of these experiments have subsequently been confirmed using ribozymes specifically directed towards c-*myb* mRNA [162] and chemically modified oligonucleotides [163]. By employing a c-*myb* "knock-down allele", expressing 5-10% on normal c-*myb* levels, Emambokus and co-workers were able to investigate the consequences of low c-Myb expression in mice [164]. The number of progenitor cells was not significantly lower than in

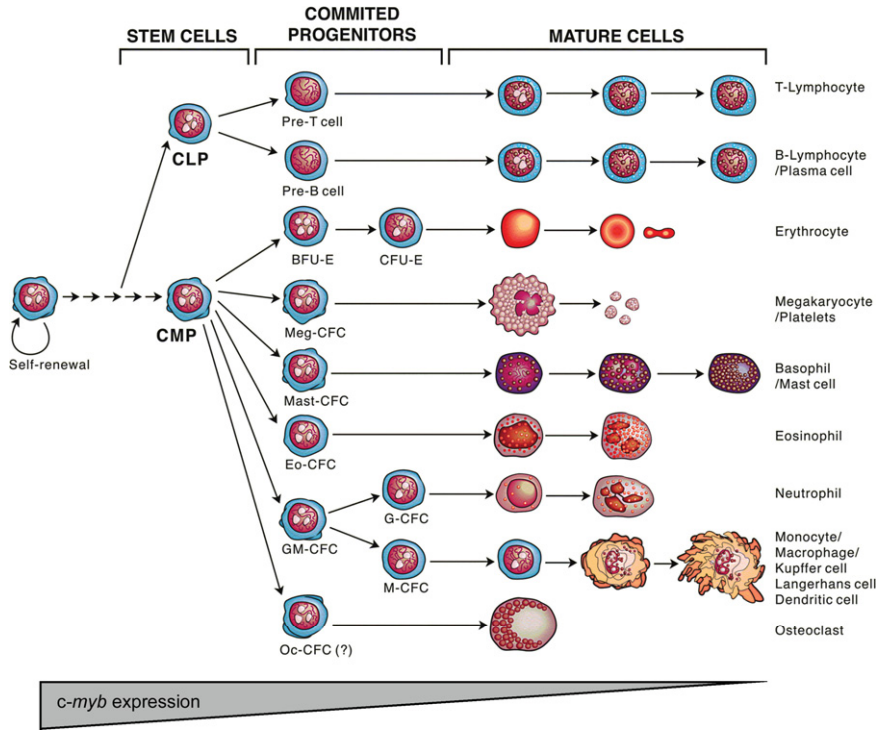

Figure 10 The hematopoiesis: c-*myb* expression is downregulated during terminal differentiation. A schematic presentation of the hematopoiesis, showing the hematopoietic stem cells and the eight major hematopoietic lineages generated by self-renewing multipotent progenitors. The Myb expression triangle is only indicative. Modified from picture in the online version of Blood Lines by D. Metcalf (www.bloodlines.stemcells.com).

wild-type mice, but the progression towards terminal differentiation was substantially altered: Erythroid and lymphoid differentiation was inhibited, leading to a shift towards more immature cells. In contrast, macrophage and megakaryocyte differentiation seemed to be favoured by low c-*myb* levels [164]. Thus, progression through key stages of the hematopoiesis seems to be dependent on distinct threshold levels of c-Myb.

Two recent screening studies in mice, where genome-wide ENU mutagenesis was employed to identify genes controlling specific hematopoietic processes, both hit the c-*myb* gene as the main affected locus. One study identified a point mutation in c-*myb* that causes thrombocytosis, megakaryocytosis, anaemia, lymphopenia, and lack of eosinophils [165], while the other study identified two point mutations that were able to rescue mice from a myeloproliferative syndrome with supraphysiological expansion of megakaryocytes and platelet production [166]. All three point mutations resulted in c-Myb proteins with lower transactivation potential, resembling the lower c-Myb levels in the knock-down study [164]. Hence a precise and stage-specific tuning of c-Myb activity and/or levels seems to be a prerequisite for the maintenance of the correct balance between proliferation and differentiation.

When myeloid cells are induced to differentiate either chemically or by cytokines, the c-*myb* expression is downregulated. This suggests that a reduction in the c-Myb level is mandatory for differentiation. In this aspect it is interesting to notice that several of the genes induced by c-Myb are markers of terminal differentiation (reviewed in [76]). One well studied example of this apparent paradox is the Myb-regulated *mim-1* gene, a differentiation marker for chicken granulocytes, which is induced by c-Myb and NF-M (chicken C/EBPβ) in erythroids and fibroblasts [96, 97, 167].

In the adult thymus of homozygous null c-*myb*/*Rag-1* chimeric mice, T cell development is blocked before oligopotent thymocytes mature into definitive T cell precursors [168]. Additional studies have suggested that c-Myb function is required for T lymphocyte development. In particular a transgene encoding a fusion protein of c-Myb DBD and the Drosophila Engrailed repressor could prevent normal thymocyte development, and resulted in animals with significant reduced number of T cells [169]. In addition, the T cells still present failed to proliferate in response to mitogen stimuli [169]. Furthermore, expression of the Myb-Engrailed dominant negative protein in thymocytes led to a marked decrease in cycling cells following β-selection [170].

Using tissue-specific deletion to abrogate c-Myb expression at distinct stages of T cell development, Bender and co-workers have identified three points at which c-Myb

activity is required for normal T cell development in the thymus: transition through the third stage of $CD4^-CD8^-$ differentiation, survival of $CD4^+CD8^+$ thymocytes, and differentiation of CD4 thymocytes [171]. These observations are supported by the fact that c-Myb is involved in regulation of genes important for T cell development; e.g. *CD4* [114, 172, 173], *TCR* δ [174, 175] and *RAG-2* [176]. A similar approach has been used to abrogate c-Myb expression in B cells. This causes a partial block of B cell development at the pro- to pre-B cell transition which results in greatly reduced production of B cells in the bone marrow [177].

1.2.4.2 Target genes

As stated in chapter 1.2.2.1 c-Myb binds to DNA at specific Myb-recognition elements (MRE). Due to the rather short Myb recognition core element, YAAC[NG/GN], potential Myb-binding sites are found in almost any random piece of DNA ≥ 1000 bp. However, several of these sites are non-functional, where one reason might be lack of availability. Hence, the presence of such a site within a promoter does not necessarily mean that the gene in question is regulated by c-Myb. Through the years quite an extensive list of c-Myb target genes has been reported, some of which have gained better support than others. Still, the genes listed in Table 1 are not the complete collection. With the new and exciting global array techniques the assembly of a complete list of c-Myb target genes should be within reach, however, experimental design, technical noise, statistics, biological confounders and cell specific gene-expression still make this a challenge. Four global studies have been reported [178-181], but it is still early days if a complete and sound catalogue of genes is the measure.

Another aspect that has become evident as new techniques have been described in parallel with a growing list of published target genes, is the necessity of defining some requirements that must be met for a gene to be claimed as a direct target of c-Myb. A classical approach to asses the validity of a proposed Myb-target gene has been (I) to identify MRE sequences in the promoter, (II) analyze DNA-binding of c-Myb to these MREs *in vitro*, (III) test the promoter segment with and without the MRE(s) in a reporter assay, and (IV) to study whether c-Myb is able to activate the potential target gene when embedded in chromatin. A criterion, less often fulfilled, is (V) to confirm that the target gene is *directly* activated by c-Myb, i.e. by demonstration of a rapid induction response, or by comparing induction in the presence and absence of protein synthesis inhibitors.

Activated Gene	Species	Function	RCG	References
mim-1	chicken	Chemotactic factor/acetyl transferase	Yes	[167]
tom-1	chicken	Membrane recruitment of clathrin	Yes	[182, 183]
Lysozyme/LYG	chicken	Enzyme degrading bacterial cell walls	Yes	[184]
A2B/ADORA2B	chicken	Adenosine receptor 2B, transmembrane	Yes	[185, 186]
C/EBPβ (NF-M)	chicken	CCAAT-box/enhancer binding protein beta	Yes	[187, 188]
Gas41	chicken	Glioma-amplified sequence 41	Yes	[189]
Mcm4	chicken	Mini chromosome maintenance 4	Yes	[186]
Pcd4	chicken	Possible tumour suppressor	Yes	[190, 191]
c-kit	hu./mo.	Tyrosine kinase receptor	Yes	[151, 152]
c-*myc*	hu./mo.	Transcription factor, proliferation-related	Yes	[144-148]
bcl2	human	Anti-apoptotic regulatory protein	Yes	[148, 153-155]
CD34	human	Hematopoietic stem cell surface protein	Yes	[192, 193]
COLIA2	human	Type I collagen α2 chain, structural protein	Yes	[194]
GBX2	human	Transcription factor, hematopoiesis	Yes	[195]
NMU	human	Neuromedin U	Yes	[196]
RAG2	human	Part of the V(J)D recombinase, lymphoid	Yes	[176]
CAI	mouse	Carbonic anhydrase I	Yes	[197]
GATA3	mouse	Transcription factor	Yes	[198]
H2A.Z	mouse	RNA Pol II histone variant	Yes	[199]
IGF-I	mouse	Insulin-like growth factor I	Yes	[200, 201]
MPO	mouse	Myeloperoxidase	Yes	[202, 203]
ADA	human	Adenosine deaminase	No	[204]
CD13	human	Digestion-related aminopeptidase	No	[205]
CD4	human	Cell surface receptor	No	[114, 172, 173]
cdc2	human	Cell cycle-related kinase	No	[206]
c-*myb*	human	See section 1.2.3	No	[207]
COX2	human	Cyclooxygenase-2, prostaglandin metabolism	No	[208]
Cyclin A1	human	Cell-cycle regulatory molecule	No	[209]
FGF-2	human	Fibroblast growth factor 2	No	[150]
hChAT	human	Choline acetyl transferase, metabolism	No	[210]
LCK type I	human	Lymphoid-spec. Src-family tyrosine kinase	No	[211]
MAT2A	human	Methionine adenosyltransferase	No	[212]
PRTN3	human	Myeloblastin, serine protease, neutrophil	No	[213]
SCF	human	Stem cell factor, hematopoietic	No	[214]
TCRδ	human	human T cell receptor δ, V(D)J recombination	No	[174, 175]
TCRγ	human	human T cell receptor γ, V(D)J recombination	No	[215]
TOPIIα	human	DNA Topoisomerase	No	[149]
TRHR	human	Thyrotropin-releasing hormone receptor	No	[216]
WT1	human	Transcription factor	No	[217]
GATA1	mouse	Transcription factor, hematopoiesis	No	[218]
NE	mouse	Neutrophil elastase, granule protein	No	[219, 220]
PAX6	quail	Transcription factor, nervous system develop	No	[221]

Repressed Gene	Species	Function	RCG	References
c-fms	hu./mo.	Macrophage colony-stim. factor receptor	No	[222]
c-erbB2	human	Tyrosine kinase receptor	No	[223]
N-ras	human	Small GTPase, intracellular signalling	No	[224]
CD4	human	Cell surface receptor	No	[114]
IgεGL	human	Ig epsilon germline, class switch recomb.	No	[225]

Table 2 Proposed target genes of c-Myb and v-Myb. The table lists reported c-Myb and/or v-Myb target genes, with stronger evidence than simple detection of Myb binding sites in the promoter. The cases where the resident chromosomal genes (RCG) have been shown to be activated by Myb are shown in the RCG-column. Repressed genes are listed in the lower part of the table.

In a recent paper we describe and discuss two new criteria which we propose should be considered [226]. In order to generate a robust core collection of direct, *bona fide* c-Myb target genes these analyses should include evaluation of (VI) endogenous promoter occupancy of c-Myb, demonstrated by ChIP, and (VII) expression of the gene affected by knockdown of c-Myb. While the latter provide strong evidence for a functional link, the former provide evidence for a direct physical link [226]. For several of the target genes listed in Table 1 one or more of these criteria are not fulfilled, or they have not been tested. Some of the reported target genes will therefore probably end up being redefined in future publications, either as indirect targets or even false positives. Nevertheless, several of the target genes have been extensively studied, and generated important knowledge of c-Myb biochemistry and biology. Some of these will be described in the following sections.

The first identified Myb target gene, ***mim-1***, was isolated using a temperature sensitive mutant of the E26 virus [167]. The *mim-1* gene product has been assigned different functions; secretable component of promyleocyte granules, hetrophil chemotactic factor, and acetyl transferase [167, 227, 228]. Endogenous *mim-1* can be induced by E26 v-Myb and c-Myb in collaboration with NF-M (C/EBPβ), but not by AMV v-Myb [96, 97]. This discrepancy has now been resolved by the findings of Mo and co-workers showing that the leukemogenic mutations in AMV v-Myb SANT/DBD eliminate the interaction with, and consequently the acetylation of, H3 tails (see section 1.2.2.1 and [54]). In contrast, the *gastrulin brain homeobox 2* gene (***GBX2***) can be activated exclusively by AMV v-Myb and not by E26 v-Myb and c-Myb [195]. While AMV v-Myb constitutively activates *GBX2* gene expression, c-Myb requires an activated signalling pathway to induce *GBX2* expression. GBX2 is a regulator of the chicken myelomonocytic growth factor (cMGF), and the constitutive production of this growth factor makes AMV v-Myb able to transform cells and induce leukemia in chicken in a growth factor independent manner [195].

Several reports have shown that **c-*myc*** could be a target gene for c-Myb [144, 145, 147, 148, 151, 229-231]. Even thought the results concerning the regulation of chromatinized c-*myc* have been contradictory, two studies have finally demonstrated that c-Myb is able to regulate the expression of resident chromosomal c-*myc*, using a conditionally active Myb-ER fusion [144] or the dominant negative Myb-Engrailed fusion protein [148]. Expression of the *MYB-ER* fusion in a myeloblastic cell line rapidly restored c-*myc* expression and thereby prevented growth arrest induced by IL-6 in nearly terminally differentiated cells [144]. This tightens the link between c-Myb, c-Myc and cell growth.

A similar approach as that used for c-*myc*, demonstrated that the anti-apoptotic oncoprotein ***bcl-2*** [148] and **c-*myb*** itself [144] are transcriptionally activated by c-Myb. Whether *bcl-2* is activated by c-Myb in all cell systems is still not known, since the expression of *bcl-2* seemed to be unaffected in DT40 c-*myb* null cells when compared to DT40 wild-type cells [231]. Concerning the c-*myb* gene, c-Myb has been show to bind to an MRE in the 5' flanking region of the gene and transactivate the promoter in fibroblasts [207]. In contrast, negative or no regulation have been observed in T cells and a myeloid cell line, respectively [232].

The finding of ***RAG-2*** as a c-Myb target gene, confirmed that Myb is essential for T cell development [168]. Chromatin IP analysis of the *RAG-2* promoter showed binding of endogenous c-Myb to a MRE in the proximal promoter. In addition Wang and co-workers showed that c-Myb was important for the activation of T cells [176]. In immature B cells cooperative binding of c-Myb and Pax-5 has been shown to activate the *RAG-2* promoter [115, 116]. *RAG-2* is encoding a component of the V(J)D recombinase, which is essential for the assembly of the antigen receptor in B and T lymphocytes. Interestingly, several other genes important for T cell development have been reported to be Myb-regulated (*CD4*, *TCR* δ and -γ; see Table 1), although the evidences for transactivation of the chromatinized genes are scarce. It should be mentioned that the cell surface receptor ***CD4*** gene is one of the few examples reported to be negatively regulated by c-Myb [114].

Recently, Maurice *at al*. has shown that c-Myb is a critical downstream regulator of positive selection of T-helper cells, from thymocytes with intermediate avidity for antigen-MHC complexes [198]. By biochemical and biological assays they demonstrated that ***GATA-3***, a critical inducer of T-helper cell fate, is a direct target c-Myb, and that c-Myb-induced *GATA-3* expression promotes transduction of the T cell receptor signal for subsequent helper T cell lineage differentiation [198].

Through the regulation of its target genes one of the main functions of c-Myb is to participate in deciding whether the cell shall continue to proliferate or start to differentiate. In this context, a set of recent papers linking c-Myb to replication is intriguing. Through their work with c-Myb target genes the group of KH Klempnauer has discovered that three different Myb targets co-localizes with origins of DNA replication [186]. Using the nascent strand abundance assay, they have been able to shown that the MREs of the ***Gas41*** [189], ***Mcm4*** [186] and ***Adora2B*** [185] genes in chicken cells all reside within areas of DNA replication origin. Still, no correlation between Myb activity and replication was observed [186]. However, DNA replication origin is known to be decondensed during replication, which may support the activation of the associated genes by c-Myb. Future studies will hopefully reveal the basis for this association.

1.2.5 c-Myb and oncogenesis

Given the central role of c-Myb in proliferation and differentiation in the bone marrow, as well as in other tissues, and the fact that the discovery of the c-*myb* gene was done in the form of two activated, leukemia-causing v-*myb* genes, it should be no surprise that the links between the c-*myb* proto-oncogene and human cancers are increasing. The genomic locus of the *MYB* gene is frequently rearranged in several human neoplasias: acute myelogenous leukemias, melanomas, and breast, colon and pancreatic carcinomas [8, 132]. In some of these cancers this involves amplification of the *MYB* gene, resulting in increased expression. Indeed increased *MYB* expression has been detected in many hematopoietic malignancies, breast and colon cancer and cancer in the upper gastrointestinal tract [233-241].

Until recently, no recurrent genomic involvement of the *MYB* locus had been reported in human malignancies. Then, in 2005 Sinclair and co-workers reported the possible involvement of *MYB* in a recurrent translocation, t(6;7)(q23;q32~36), coupled to childhood T cell acute lymphoblastic leukemia (T-ALL; [242]). This translocation, found in 7 patients, affected the genes *MYB* and *AHI1*, which were shown to reside within the break-point region. Still, this translocation was not correlated with changes in *MYB* expression [242]. The same translocation was earlier this year mapped in detail to t(6;7)(q23;q34), showing that in all cases the translocation places *MYB* in the vicinity of the *TCRB* (T cell receptor β) regulatory sequence [243]. Quantitative real-time PCR demonstrated that the *TCRB-MYB* translocation indeed deregulated the expression of *MYB* which showed significantly higher levels than in other T-ALL patients. With more than 7% of the patients

studied having this translocation, the authors were able to define a new T-ALL subtype in very young children [243].

In parallel with the identification of the *TCRB-MYB* translocation another and even more common perturbation of the *MYB*-locus was revealed. In two independent studies more than 11% of the T-ALL patients studied (9 of 107 cases; 13 of 84 cases) turned out to have a short local duplication of the *MYB* gene [243, 244]. The MYB^{dup} patients like the *TCRB-MYB* patients had elevated *MYB* mRNA levels, but in this case it correlated with the duplication. In one of the studies MYB^{dup} was shown to concur with mutations in the *NOTCH1* gene in all cases [244]. Lahortiga and co-workers also demonstrated that knockdown of *MYB* in T-ALL MYB^{dup} cell lines (RPMI-8402 and MOLT-4) irreversibly initiated T cell differentiation. Furthermore, the combined inhibition of *MYB* and *NOTCH1* by RNAi strongly affected proliferation and survival in a synergistic manner [244]. This suggests that *MYB* might be a therapeutic target in human T-ALL.

c-Myb has also been implicated in cancer of the breast [235, 236] and *MYB* expression correlates strongly with ER positive breast cancer [235]. Earlier this year Drabsch and co-workers showed that this is due to ER acting directly on the *MYB* gene to relieve attenuation caused by a stem-loop structure in the first *MYB* intron [245]. The investigators further showed that *MYB* is required for the proliferation of ER^+ breast cancer cells, as they were able to halt cell proliferation by knocking down *MYB*. This demonstrates that c-Myb is an effector of estrogen/ER signalling and provides evidence for a functional role of *MYB* in breast cancer [245].

Altogether the recent advances in the field of c-Myb and oncogenesis have strengthened the link between c-Myb aberrations, increased expression and human cancers. This has positioned the *MYB* gene as a potential cancer drug target where the aim would be to halt its expression or reduce its activity[2]. With its key role in hematopoiesis and leukemogenesis, unravelling the molecular mechanisms of the c-Myb protein is paramount.

[2] www.drugresearcher.com/news

1.3 SMALL UBIQUITIN-RELATED MODIFIER - SUMO

The small ubiquitin-related modifier, SUMO, was identified in mammalian cells in 1996, where it was found to be covalently linked to the GTPase activating protein RanGAP1 [246, 247]. Since its discovery, the number of SUMO targets has grown. Moreover, SUMO conjugation has been shown to be essential for cell viability [248-250]. Presumably, SUMO is just as important for the cell as its close relative ubiquitin, and perturbations in the SUMO system, as well as in the ubiquitin system, have been implicated in several different diseases and cancers (reviewed in [251]; see section 1.3.4). SUMO-related mechanisms are seemingly also an important part of c-Myb biology, and since two of the papers presented in this thesis deals with SUMO-regulation of c-Myb, a chapter dedicated to this nuclear protein has been included.

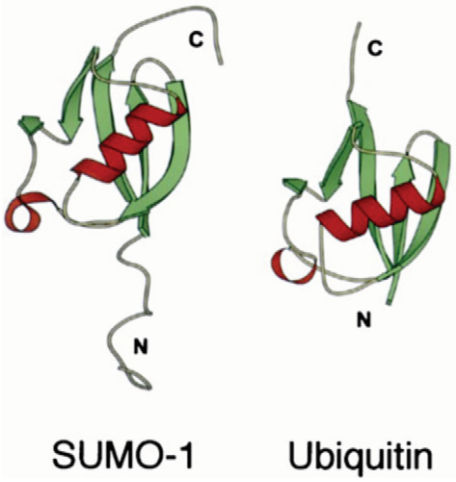

Figure 11 The ubiquitin-fold. Ribbon diagrams highlight the similarity of the three-dimensional structures of SUMO-1 and ubiquitin [252]; from N- to C-terminus; ββαββαβ. Secondary structure elements are indicated with colour; β sheets (green) and α helices (red). Modified from illustration in [253].

1.3.1 Ubiquitin-like proteins

Since the discovery of ubiquitin in the mid-1970s, an entire family of small proteins related to ubiquitin has been identified and classified. Based on multiple sequence alignments, 3D threading, and dissection of the enzymatic mechanisms it has been possible to add about a dozen ubiquitin-like proteins (Ubls) to the list, e.g. ISG15, Fat10, Nedd8 and SUMO (reviewed in [254]). Despite sharing only medium sequence similarity, all the Ubls possess similar three-dimensional structure; the Ubiquitin- or the β-grasp fold (ββαββαβ; Fig 11; [255]).

In general all Ubls are conjugated C-terminally to an internal lysine in the protein substrate, making an isopeptide bond. However, there are some exceptions [256]. The C-terminal amino acid residue of the Ubls, in which the carboxyl group is the site of substrate attachment, is always a glycine. In addition, the conjugation mechanism is similar for all the

Ubls studied (Fig 12; [253, 254]). In short, **I:** exposure of the C-terminal glycine by proteolytic cleavage, **II:** activation of the Ubl by an activating E1 enzyme, **III:** transfer of the Ubl to an E2 conjugating enzyme, which either **IVa:** forms a four protein complex with the Ubl, the substrate and an E3 ligase, or **IVb:** transfer of the Ubl to the E3 ligase, and finally **V:** conjugation of the Ubl to the substrate (see section 1.3.2.2 for details). The Ubl can later be cleaved off by an isopeptidase, often the same protease as in step I, and used to modify other substrates (reviewed in [253]).

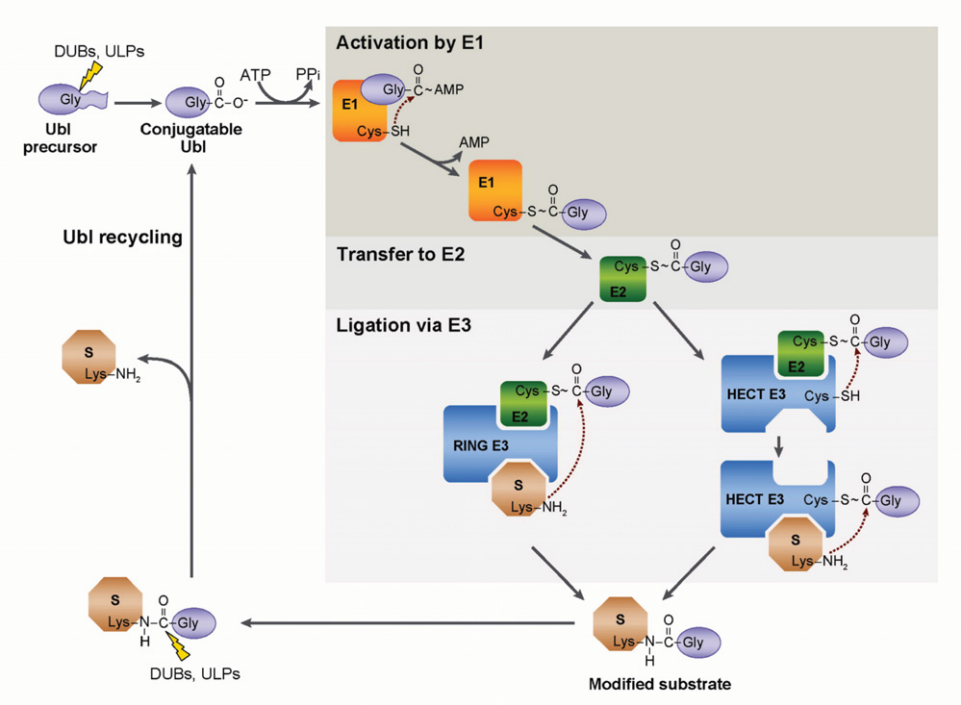

Figure 12 A generalized Ubl-conjugation pathway. Precursor Ubls are processed by DUBs (deubiquitinating enzymes) or ULPs (Ubl-specific proteases) to expose the C-terminal glycine in the mature Ubl. The processed Ubl is activated with ATP by E1 Ubl-activating enzyme. This creates a thioester linkage between the Ubl and E1. The Ubl is transferred to the catalytic cysteine of the E2 Ubl-conjugating enzyme, and can then be ligated to a substrate with the aid of an E3 Ubl-protein ligase. The RING E3s catalyze modification by binding simultaneously the Ubl-E2 thioester complex and the substrate to be modified. HECT E3s catalyze substrate ligation in two steps. First, the Ubl is transferred to a catalytic cysteine of the HECT E3. Then, the E3-Ubl thioester complex transfers the Ubl to the substrate. The DUBs and ULPs can remove Ubls from substrates. Taken from [254].

Several of the Ubls function as important regulators of various cellular functions including transcription, DNA repair, signal transduction, autophagy, and cell cycle control [257-259]. Even though the Ubls are relatively small proteins (Ubiquitin: 76 aa; SUMO: ~100 aa), a Ubl moiety may alter the biochemical properties of a substrate considerably. One such alteration is the introduction of new protein interaction surfaces. So far this aspect

of Ubl biochemistry has been best studied for ubiquitin. In addition to being conjugated, ubiquitin can bind non-covalently to proteins harbouring ubiquitin-interaction/binding motifs (UIMs/UBMs). These motifs have been given a lot of attention the last years (reviewed in [260]), and reports on interaction motifs for other Ubls have now started to appear in the literature (see chapter 1.3.3 and [261, 262]).

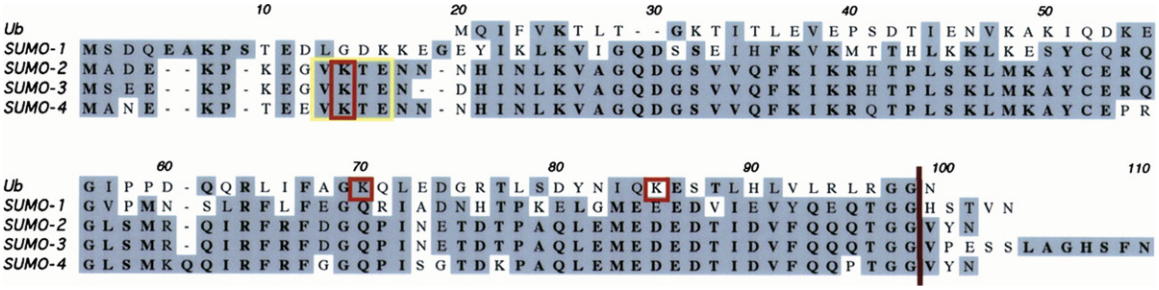

Figure 13 SUMO is highly related to ubiquitin. Multiple sequence alignment of human ubiquitin and the four human SUMO homologues. Identities are indicated in bold and similarities are shaded. A consensus motif for sumoylation present in SUMO-2, -3 and -4 is boxed in yellow, with the SUMO acceptor lysine boxed in red. Ubiquitin lysine 48 and 63, which serve as common sites for ubiquitin polymerization, are also boxed in red. The site of cleavage for production of mature proteins with C-terminal glycine-glycine residues is indicated with a vertical line. Modified from illustration in [253].

1.3.1.1 SUMO isoforms

Beside ubiquitin itself, SUMO is by far the Ubl given the most attention. Today, four SUMO isoforms have been described in mammals; SUMO-1, -2, -3 and -4. The SUMO family consists of nuclear proteins of around 100 amino acids, which are ubiquitously expressed [253], except SUMO-4 which has a restricted pattern of expression [263]. SUMO-2 and -3 are highly similar, differing from one another by only three N-terminal residues, and have yet to be functionally differentiated [264, 265]. They form a distinct sub-family, often referred to as SUMO-2/3, and are only 50 % identical to SUMO-1. This difference is actually as large as the one fond between ubiquitin and Nedd8 [254]. The fourth and most recently described SUMO isoform, SUMO-4, is ~ 85 % identical with SUMO-2/3. Due to a proline residue positioned six amino acids from the C-terminus, SUMO-4 cannot be matured by the SUMO specific proteases (Fig 12; see chapter 1.3.1 and 1.3.2.2), and hence it is unlikely to be conjugated. Still, it might function as a dominant negative SUMO [266]. In this context it should be noted that among the tissues investigated the highest level of *SUMO-4* expression was seen in kidney [263]. However, as evaluated by large-scale microarray analysis of the human transcriptome (Novartis Research Foundation's GNF SymAtlas; [267]), the *TAB2* gene, in which the *SUMO-4* gene resides in

an intron, is also highly expressed in dendritic cells, whole blood, and in hematopoietic cells from both the myeloid and lymphoid lineage.

Apart from the difference in primary structure, there are other dissimilarities between SUMO-1 and SUMO-2/3. Virtually all SUMO-1 in the nucleus is engaged in conjugates, while for SUMO-2/3 there seems to exist a free pool that is incorporated into high molecular weight complexes when the cells are exposed to oxidative-, heat- or genotoxic stress [264]. The ability of ubiquitin to form polymeric chains is critical for many biological activities of this protein, like the interaction with the proteasome. In contrast, SUMO is generally thought to function as monomers. Nevertheless, SUMO-2/3 has been observed to form chains *in vitro* [265]. SUMO-2/3 and -4 harbours a consensus motif for sumoylation in the N-terminus (yellow box Fig 13; see section 1.3.2.1), which might facilitate this polymerization. Nevertheless, the functional significance of SUMO chains *in vivo* has yet to be established [265, 268-270]. It is, however, possible that SUMO polymerization or other post-translational modifications might add complexity to SUMO in mammalian cells.

The SUMO protein is like ubiquitin evolutionary well-conserved, and homologues have been identified in many eukaryotes, including yeast. However, in contrast to the four SUMO isoforms found in mammals, yeast only expresses one, termed Smt3. Smt3p is 48 % and 44 % identical to human SUMO-1 and SUMO-2/3, respectively, but still has the ubiquitin-fold [271].

1.3.1.2 SUMO structure

The SUMO-1 amino acid sequence shares only ~ 18 % identity with ubiquitin, still their three-dimensional structures are highly homologous. SUMO-1 and ubiquitin share the characteristic ββαββαβ-fold, also known as ubiquitin- or the β-grasp fold ([255]; Fig 11), and the C-terminal di-glycine motif (Fig 13) which is necessary for interaction with E2 and creation the isopeptide bond [272]. Unique for the SUMO peptides is the 16-20 amino acid long and flexible N-terminal tail (Figs. 11 and 13). In addition, the distribution of positively and negatively charged surface residues on SUMO is highly different from ubiquitin. These distinctions are probably the underlying reason for their different biological functions, as well as enzyme and substrate specificities (reviewed in [253]).

Given the difference in primary structure between SUMO-1 and SUMO-2/3, it is obvious that this is also reflected in three-dimensional space. All three isoforms form a hydrophobic groove, surrounded by basic residues, between the second β-strand and the

first α-helix (from the N-terminal; Figs 11 and 16). However, the basic residues are positioned slightly differently in the three isoforms. While the hydrophobic groove has been shown to be important for interaction with other proteins [273, 274] and correct positioning of SUMO during conjugation [275], the different positioning of the surrounding basic residues has been implicated in advocating specificity both in protein-protein interactions ([276]; see chapter 1.3.3) and during selection of SUMO paralogues for conjugation [277].

1.3.2 SUMO-conjugation

The three best studied SUMO isoforms; SUMO-1, -2, and -3, are all conjugated to lysines positioned in a certain context. Apart from the fact that these lysines must be exposed on the surface of the targeted protein, several additional constraints exist, some of which have been deduced in the last couple of years.

1.3.2.1 Consensus sequence

Analyses of different SUMO substrates pointed to the existence of a sumoylation consensus motif; ψKXE both in mammals [278, 279] and yeast [280], where ψ is a hydrophobic or bulky residue and X is any amino acid. This sequence can bind directly to the E2 SUMO-conjugating enzyme Ubc9 ([278, 281]; see section 1.3.2.2). Recently, the consensus has been extended by Yang and co-workers showing the dependency of negatively charged residues located directly C-terminal of this motif [282]. The requirement of the negatively charged amino acids in the extended motif; NDSM (negatively charged amino acid-dependent sumoylation motif), has also been correlated to increased affinity between the substrate and Ubc9 [282].

The negatively charged amino acids can also be compensated with residues that can be phosphorylated, like in the PDSM (phosphorylation-dependent sumoylation motif) suggested by Hietakangas and co-workers [283]. This adds a potential layer of regulation to SUMO-conjugation: In proteins harbouring a PDSM, a phosphorylation cue might fulfil the requirement for negative charge in the sumoylation motif and subsequently trigger SUMO-conjugation [283]. The SUMO-acceptor lysine K527 in human c-Myb ($IK_{527}QEVES_{532}$ PTDKS) is part of a potential PDSM, as S532 can be phosphorylated [84-86]. But since this motif contains two other negatively charged glutamate residues, phosphorylation of S532 is not essential for sumoylation to occur (V. Matre unpublished results).

Interestingly, there are also reports of SUMO being conjugated to sites not conforming to the consensus [284]. Furthermore, some proteins not even harbouring

sumoylation consensus motifs have been shown to be sumoylated, as well as proteins where all acceptor lysines have been mutated [285-287]. Thus, there still seem to be novel sumoylation motifs waiting to be defined.

1.3.2.2 Mechanism

The sumoylation process is highly dynamic and reversible. By the aid of proteases, the SUMO protein can be deconjugated and reconjugated to substrate proteins in several consecutive rounds. As indicated in chapter 1.3.1 this five step process includes four different enzymes; a SUMO isopeptidase, a SUMO E1 activating enzyme, a SUMO E2 conjugase and a SUMO E3 ligase.

SUMO proteases

In the first step the SUMO precursor protein has to be trimmed at the C-terminus, to expose the Gly-Gly motif. This is done by proteases which contain two different activities; an endopeptidase activity used to mature SUMO precursors, and an isopeptidase activity used to remove conjugated SUMO from target proteins. The SENPs (Sentrin-specific protease; Sentrin: original name for SUMO) constitute a family of SUMO-proteases harbouring these activities. Human cells express seven different SENPs (SENPs -1, -2, -3, -5, -6, -7, and -8), where SENP8 actually is a Nedd8 protease (reviewed in [288]). With their different tissue expression, different subcellular localization, and different SUMO isoform preference, these enzymes are thought to augment specificity in the sumoylation pathway (reviewed in [289]).

SENP1 and -2 constitute the best characterized SENP subfamily. SENP1 is localized in the nucleoplasm and deconjugates a large amount of sumoylated proteins, while SENP2 is associated with the nuclear pore. SENP1 has a preference for SUMO-1 [290, 291], while SENP2 seems to have a preference for SUMO-2/3 [292]. However, they are both capable of processing and deconjugating all three isoforms. SENP3 and -5 on the other hand are only able to deconjugate SUMO-2/3. Whether or not they also can process one ore more SUMO isoforms is not known [289].

SUMO E1 activating enzyme

The human SUMO E1 activating enzyme consists of a hetrodimer named SAE1/SAE2 [293, 294]. To initiate the SUMO modification reaction SAE1/SAE2 catalyzes the formation of adenylated SUMO in which the C-terminal carboxyl group is covalently linked to AMP. Breakage of the AMP linkage is followed by formation of a covalent intermediate in which

the C-terminal carboxyl group of SUMO forms a thioester bond with the sulphydryl group of a cysteine residue in SAE2 (Fig 12). It has been shown that the adenovirus protein Gam 1 can lower sumoylation through inhibiting the E1 activity by blocking the formation of the SUMO-E1 thioester complex [295]. This results in transcriptional activation of some promoters, and implies that the sumoylation machinery might also be a target for other viruses [295].

SUMO E2 conjugating enzyme

In the third step of the sumoylation cycle SUMO is transferred from E1 to a cysteine residue in the active site of the SUMO E2 conjugating enzyme Ubc9, forming an E2-SUMO thioester intermediate. This intermediate functions as a SUMO-donor in the last step where SUMO is transferred to the amino group of the acceptor-lysine in the target protein [296, 297]. Ubc9 is the only Ubl E2 conjugating enzyme that directly recognizes substrate proteins. The conjugase can bind to the sumoylation consensus sequence ΨKXE [278, 282] and catalyze the formation of an isopeptide bond between the C-terminal carboxyl group of SUMO and the ε-amino group of the lysine in the sumoylation motif. In addition Ubc9 contains an N-terminal domain which binds directly to SUMO and facilitates the transfer of SUMO from the E1 enzyme [298].

While there have been reported more than 30 Ubiquitin E2 conjugases, only one is known for SUMO [299]. Ubc9 is therefore essential for SUMO-conjugation, and by knocking it out, all sumoylation is abrogated. Ubc9 null embryos progress to implantation, probably due to the exictence of maternal protein during preimplantation stages, but die after the early postimplantation period [250].

SUMO E3 ligase

Even though SAE1/SAE2 and Ubc9 is sufficient for SUMO modification *in vitro*, it is rather inefficient, and additional components are needed to accelerate this reaction [300, 301]. The SUMO E3 ligases are such factors, and they promote transfer of SUMO from the E2 conjugase to specific substrates. Several SUMO E3 ligases have been identified, most of which seem to employ the “IVa mechanism” described in chapter 1.3.1, forming a four protein complex with SUMO, Ubc9 and the substrate. This is typical for the so-called RING domain-containing E3s (reviewed in [259]).

Today, three families of SUMO E3 ligases are recognized; the **PIAS** (protein inhibitors of activated STAT) proteins, **RanBP2**, and the PcG (polycomb group) protein

Pc2 [269, 302, 303]. In humans the PIAS proteins (PIAS1, PIASxα, PIASxβ, PIASy and PIAS3; [304-306]) are characterized by a central cysteine-rich SPRING (Siz/PIAS RING) domain [307]. The substrate specificity of these E3 enzymes is not known, but knockout studies in mice suggest a certain amount of redundancy [308, 309]. In the case of c-Myb, PIASy has been shown to induce SUMO-1 conjugation, while PIAS3 has been shown to be important for stress-induced SUMO-2/3 conjugation [81, 83]. Lately, several of the PIAS proteins have been shown to affect the activity of transcription factors in an E3-independent manner [310-312].

The nuclear pore protein RanBP2 (a.k.a. Nup-358) is not a SPRING-containing E3 ligase, and is in general not related to the Ubl E3s. RanBP2 has been shown to accelerate the conjugation of SUMO-1 to Sp100 and HDAC4 [269, 313] and SUMO-2 to PML [277]. RanBP2 is responsible for localizing sumoylated RanGAP1 to the nuclear pore complex [314, 315], and the four-protein conjugation complex involving RanGAP1, RanBP2, Ubc9 and SUMO-1 has been solved [275].

The polycomb chromatin-modifying complex mediates transcriptional repression, and the Pc2 component of this complex has been shown to possess SUMO E3 ligase activity. Like RanBP2, Pc2 is neither a RING, nor a HECT-containing E3. Still it has the capacity to bind both Ubc9 and its substrate protein [316]. One of the best described substrates for Pc2-dependent SUMO modification is the transcriptional co-repressor CtBP [302]. Ectopic expression of Pc2 leads to co-localization of SUMO and Ubc9 in PcG bodies, implying that PcG bodies might be an important site for sumoylation [302]. Recently, it has been shown that during DNA damage response the Pc2 E3 activity is enhanced as a result of phosphorylation carried out the homeodomain interacting protein kinase 2 (HIPK2). This leads to increased sumoylation of HIPK2, which enhances its ability to mediate transcriptional repression during DNA damage (reviewed in [317]).

1.3.2.3 Biological consequences

As the field of SUMO has expanded it becomes more and more evident that the small-ubiquitin related modifiers are involved in many nuclear processes. SUMO modulates components involved in nuclear transport, DNA repair, chromosome organization, signal transduction, formation of nuclear structures, and gene expression. Here I will focus on the role of SUMO in gene expression, but first briefly mention the other processes in which SUMO participates.

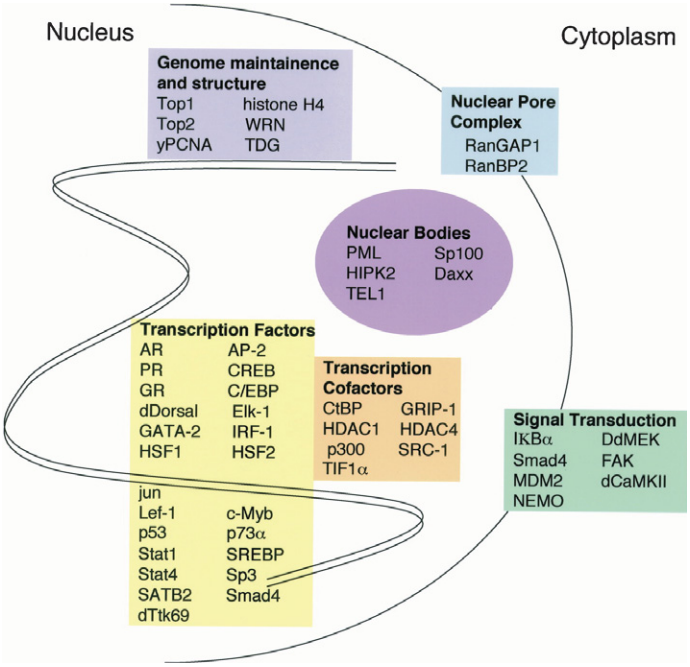

Figure 14 Targeted by SUMO. A selection of identified sumoylation substrates, arranged by function and localization. Several of these proteins are involved in transcriptional regulation, organization of chromatin, maintaining of genome integrity, signal transduction and formation of nuclear bodies. Taken from [253].

Nuclear transport

Sumoylation has been shown to regulate the nuclear translocation of several proteins [287, 318-320]. RanGAP1 is a good example of this function: Unmodified RanGAP1 is cytoplasmic, while sumoylated RanGAP1 is associated with the nuclear pores in the nuclear envelope [314, 315]. Interestingly several sumoylation consensus motifs have been shown to reside within nuclear localization signals [286, 321], and it has been suggested that most SUMO substrates are sumoylated when translocated to the nucleus [322]. As mentioned in chapter 1.2.3.2 reported data have indicated that cytoplasmic sumoylation of c-Myb by TRAF7 can sequester c-Myb in the cytoplasm, thereby lowering c-Myb transactivational potential [110]. Although exciting, we have not been able to substantiate these data using c-Myb-SUMO fusion constructs (AH. Alm-Kristiansen unpublished data).

DNA repair and chromosome organization

Several proteins involved in DNA repair and DNA replication have been shown to be modified by SUMO, including PCNA (proliferating cell nuclear antigen), the RecQ-type helicase WRN, Topoisomerase I and II, and thymine-DNA glycosylase TDG (reviewed in [323]). Sumoylation of a ubiquitin-acceptor lysine has been demonstrated to counteract ubiquitination-dependent PCNA repair activity in undamaged cells. Furthermore, sumoylation of PCNA is increased during S phase, and is therefore thought to play a role in replication [284]. TDG removes thymine and uracil from mispaired G-T and G-U base pairs.

Dissociation of TDG from DNA after base removal is an essential step in completing the repair of such damages. Sumoylation of TDG has been shown to abrogate DNA-binding, probably mediated by conformational changes in TDG promoted by intra-molecular, non-covalent SUMO binding (see chapter 1.3.3; [324-326]).

SUMO plays an important role in protecting chromosome structure and function. All the components of the sumoylation machinery (E1, E2, E3 and SENPs) have shown genetic associations with the regulation of chromosome condensation, cohesion, and mitotic/meiotic chromosome separation [327]. Furthermore, Ubc9 knock-out mice, which die *in utero* post implantation, have severe chromosomal defects. These mice show aberrant chromosomal condensation and segregation, and abnormal nuclear morphology [250].

Signal transduction

Several lines of evidence point in the direction of SUMO being involved in signal transduction. Moreover, SUMO conjugation itself might be controlled by intracellular signals. One way for the cell to transmit signals, e.g. stress signals, is through phosphorylation. As mentioned, a phosphorylation dependent sumoylation motif has been described ([283]; see section 1.3.2.1). In the same paper the transactivation capacities of the heat-shock factors HSF1 and HSF4b, harbouring PDSMs, were demonstrated to be repressed as a result of phosphorylation-dependent sumoylation [283].

Another example of a link between sumoylation and cellular signals is the significant increase in SUMO-2/3 conjugation following heat-shock and oxidative stress [264]. Prolonged hypoxia induces CREB sumoylation [287], while DNA damage-inducing agents augment SUMO conjugation of the IκB kinase-regulator NEMO [320]. Decreased phosphorylation of Promyelocytic Leukemia Protein (PML) after mitosis is necessary for increased sumoylation of PML and assembly of the PML nuclear bodies (PML-NBs) during interphase [328].

Some proteins are modified by sumoylation, ubiquitination and acetylation on the same lysine leading to different outcomes [284, 329, 330]. However, competition for the same acceptor lysine is probably only one aspect of this interplay. Sumoylation has been suggested to increase deacetylation of histone H4 [331], while several histone acetyl transferases (HATs) and deacetylases (HDACs) change localization and/or activity as a result of SUMO conjugation [313, 332, 333]. One example is HDAC-1, for which the deacetylase activity is stimulated by sumoylation [333].

Regulation of transcription

Posttranslational modification by SUMO has diverse effects on stability, localisation and activity of transcriptional regulators. Proteins involved in transcription, ranging from sequence-specific transcriptions factors, to co-activators and co-repressors, from histone modifying enzymes and chromatin remodelling factors to proteins in the general transcription machinery, have been shown to be sumoylated [253, 334]. Sumoylation of DNA-binding transcriptional regulators generally leads to a repression of their transactivational potential. This has been demonstrated for e.g. Elk-1 [335], C/EBPβ [336], AR [337] and c-Myb [81, 82]. TCF-4 (T cell factor-4; [338]) and Ikaros [339] are some of the few examples suggested to be positively modulated by sumoylation.

In contrast to ubiquitination that mainly marks proteins for proteasomal degradation, sumoylation has been linked to stabilization of several proteins. This has been shown to be achieved either by directly competing with ubiquitin for acceptor lysines as in the case of IκBα [329], or through more independent and indirect mechanisms as reported for Smad4 and Huntingtin [340, 341]. Ubiquitination of transcription factors has been proposed to act as a licensing mechanism that stimulates transcriptional activity, but at the same time targets the factors for proteasomal degradation [323, 342, 343]. When thinking of SUMO as an anti-ubiquitin, the opposite would be the case; repression and stabilisation [323]. Indeed, this effect has been observed in transrepression with sumoylated PPARγ. Sumoylated PPARγ interacts with the N-CoR-HDAC3 complex and thereby blocks subsequent recruitment of the ubiquitination machinery responsible for clearing the promoter [344].

The predominant repressive effect of sumoylation is in most cases linked to the recruitment of factors inhibiting transcription. Such factors include co-repressors like the HDAC family [332, 335] and Daxx [345, 346]. Yang and Sharrocks have observed that sumoylation of Elk-1 promotes its association with HDAC2, decreased histone acetylation, and repression of an Elk-1 target gene [335], while Lin and co-workers have shown that Daxx represses the transcriptional activity of sumoylated GR as well as AR, Smad4, and CBP [346]. With the discovery of SUMO-binding motifs in several SUMO-interacting proteins, including HDACs and Daxx, there are reasons to believe that co-repressors will be tethered even tighter to SUMO-modification (see chapter 1.3.3; [273]).

Another interesting aspect of SUMO-conjugation is that it seems to be disrupting transcriptional synergy on promoters with multiple response elements. In 2000 Iniguez-Lluhi and Pearce identified a short protein motif in the glucocorticoid receptor (GR) that

mediated “synergy control” (SC) [347]. Mutations of this site unleashed a strong synergistic behaviour of GR at compound promoters, but not at single response elements. Later it was shown that the SC motif was indeed a SUMO-conjugation site, and that the control of synergy was caused by sumoylation of the factor [348, 349]. This role of SUMO has been extended to other transcription factors, such as Ets-1 and C/EBPα [347, 350]. The third paper presented in this thesis addresses the SC aspect in c-Myb and Myb-dependent gene activation (see Paper III).

A conundrum within the field of SUMO research is the paradox of stoichiometry. Even though only a small fraction of a given target protein seem to be sumoylated at any given time, the biological consequences of abrogating SUMO-conjugation do not appear proportionate [259]. Here c-Myb may serve as an example: Even though as little as 5-10 % of the c-Myb species are conjugated to SUMO as judged from a Western analysis, mutating the acceptor lysines may result in a 20-fold increase in activity. Several models have been presented trying to account for this discrepancy [259], but till date none of them has been confirmed. Proposals include SUMO as a dynamic element in the transcriptional process, SUMO as an assembly factor, necessary only temporarily while a complex is formed, or SUMO as relocalization tag, changing the localization of its target protein and hence its activity [259]. Also the SUMO-governed synergy control and loss of this, leading to a more than additive effect on transcription, might be a candidate explanation.

Concerning SUMO-dependent relocalization of transcription factors, recent work on the tumour suppressor PML have provided new insight into this aspect of SUMO biology. As stated above, the PML protein has the ability to polymerize into what is known as the PML nuclear bodies (Fig 15). The PML-NBs dynamically change its morphology during the cell cycle and in response to cellular stresses [328]. SUMO-conjugation of the PML protein is paramount for the bodies to nucleate [351, 352], and mutations of the SUMO-acceptor lysines in PML or ectopic expression of SENPs results in delocalization of PML and PML-NB associated factors, e.g. CBP and Sp100, within the nucleus [352, 353]. Recently, it was demonstrated that a motif in PML, mediating non-covalent interactions with SUMO (SUMO-binding motif; SBM), is just as essential for PML-NB formation as SUMO-conjugation. [354]. It is now thought that SUMO:SBM interactions are the ‘glue’ in the PML-NBs (Fig 15). In addition, the co-repressor Daxx, which harbours an SBM, is recruited to PML-NBs in an SBM-dependent manner, by binding to PML-conjugated SUMO moieties. This relieves Daxx-mediated transcriptional repression [346]. Presumably,

SUMO-conjugation and SUMO-binding are the underlying mechanisms controlling the recruitment of many PML-associated factors to PML-NBs (Fig 15; [355]).

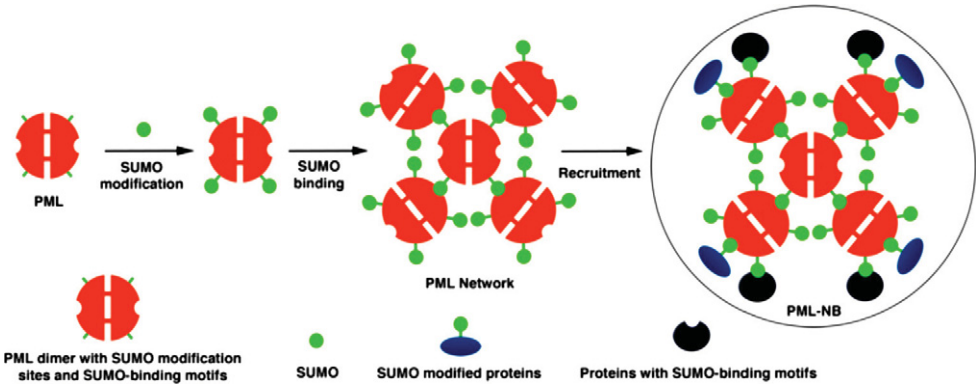

Figure 15 SUMO: The glue that binds. PML forms a homodimer, with each subunit containing multiple SUMO modification sites (green lines) and a single SUMO-binding motif (notch). Intermolecular interactions, mediated by SUMO and SUMO-binding motifs, nucleate the formation of a PML network that is able to recruit an assortment of other SUMO-modified proteins and proteins with SUMO-binding motifs. Adapted from [355].

1.3.3 Non-covalent SUMO-binding

As evident from the preceding chapter, non-covalent SUMO-binding has been observed for some nuclear factors. In 2004 Song and co-workers showed that a small hydrophobic patch; V/I-X-V/I-V/I, is the minimal motif needed for SUMO-interaction [273]. This only partly fitted with a motif proposed earlier; h-h-X-S-X-S/T-a-a-a, by Minty and co-workers ([356]; h: hydrophobic residue, a: acidic residue). However, with a study of SUMO-binding motifs in yeast (I/V-I/L-I/L-X_3-D/E/Q/N-D/E-D/E; [357]) and the work of Hecker and co-workers [276], the suggested consensus sequences were harmonized; V/I-X-V/I-V/I-a-a-a. Furthermore, the motif was proven to be able to bind SUMO also when reversed; a-a-a-V/I-V/I-X-V/I/L [274, 276]. In mammalian proteins the hydrophobic core allows for SUMO-binding, while the negatively charged amino acids surrounding the core augments SUMO-isoform specificity and probably also the orientation of the binding. SUMO-1 seems to be more dependent on such an acidic stretch for efficient binding, while SUMO-2/3 may bind in its absence [276]. This correlates with the basic residues positioned slightly different between the second β-strand and the first α-helix in the different SUMO isoforms [276]. As for the phosphorylation-dependent sumoylation motifs, phosphorylated amino acid residues can compensate for the lack of acidic residues in potential SBMs. Still, this only changes the dynamics and strength of the interaction, and so far no signal-dependent SBM has been revealed [276].

Motifs binding non-covalently to ubiquitin have also been known for some years (reviewed in [260]). However, in contrast to the similarities found between ubiquitin and the SUMO family concerning three-dimensional structure and mechanism of conjugation, the non-covalent binding properties of these modifiers differ. As can be seen in figure 16, interacting proteins contact ubiquitin and SUMO on opposite sites of the molecules. Moreover, the affinities of SBMs for SUMO are in the 2-3 μM range, while those of UBM for ubiquitin are weaker and in the range of 10-500 μM [261]. Finally, only one SBM has been described so far, while there are more than 15 different known UBMs [260]. The number of SBMs might however increase with future work.

The recognition of the SUMO-binding motifs (SBM) has provided new insight into the interplay between sumoylation and SUMO-binding, with the tumour suppressor PML as one of the best examples [354]. A question worthwhile asking is whether the SBM-SUMO interaction provides enough specificity in the interaction between SUMO-conjugated and SUMO-binding factors. If so, all sumoylated proteins could potentially interact with every factor holding an SBM. Most likely additional contacts between the substrate proteins are needed. The non-covalent SUMO-binding properties of c-Myb are addressed in Paper IV in this thesis.

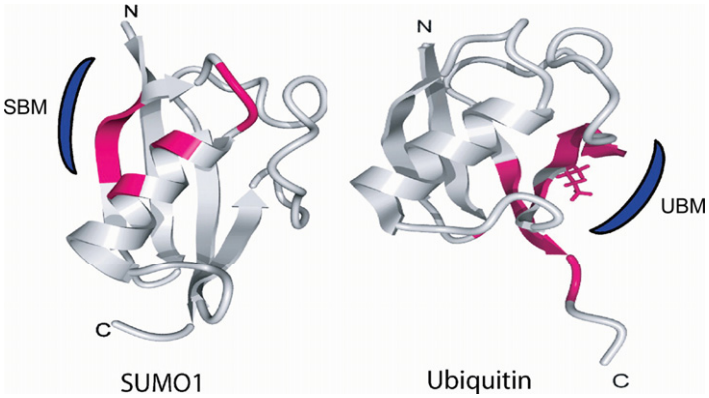

Figure 16 SUMO and Ubiquitin: same family, different binding sites. Comparison of the SBM-binding site on SUMO-1 and the UBM-binding site on ubiquitin. The SBM binds to an area defined by the second β-strand and the first α-helix of SUMO, whereas the UBM binds to a group of amino acids of the third and fourth β-strands of ubiquitin. Modified from illustration in [276]

1.3.4 SUMO pathology

As the field of SUMO has evolved, several diseases have been linked to perturbations in the SUMO system [251]. Pathogen infections, neurodegenerative conditions and cancer have been associated with dysregulation of SUMO modification enzymes or disruption of sumoylation by mutations in substrate proteins. Pathogenic viruses and bacteria exploit the

host sumoylation apparatus during infection and replication [358]. YopJ, which codes for a SUMO-protease homologue, and Gam 1, which inhibits E1 activity, are two such examples [295, 359].

SUMO-3 seems to be regulating the processing of amyloid precursor protein (APP) to amyloid β peptide (Aβ) in hippocampal neurons. Increased production or deposition of Aβ is involved in the development of Alzheimer disease (AD; [360]). Low expression of SUMO-3 or expression of SUMO-3 mutants, cause an increase in production of Aβ from APP [361]. Interestingly, the SUMO-3 distribution seems to be limited to the neuronal cytoplasm in the AD hippocampus in contrast to non-demented brains, where SUMO-3 is both cytoplasmic and nuclear. The PIAS3 expression has been found to be elevated in several types of cancer including lung-, breast-, prostate- and brain tumours [362], while an increased expression of SENP1 has been demonstrated in prostatic intraepithelial neoplasia and prostate cancer, but not in normal prostate [363]. Moreover, since SENP1 is a strong activator of AR-dependent transcription, this ultimately leads to an increase in cellular proliferation in the prostate [363].

The fusion protein PML-RARα, caused by translocation, cannot be sumoylated, hence it cannot form PML-NBs. Moreover, this translocation has been shown to induce acute promyelocytic leukemia (APL; [364]). Retinoic acid and arsenic trioxide treatment induces degradation of the PML-RAR chimera and reorganizes PML nuclear bodies by enhancing sumoylation of the PML moiety [365]. Thus, arsenic trioxide is used routinely in the treatment of APL patients [366]. These few examples illustrate the significance of a well-functioning SUMO-system, and indicate that SUMO-related factors might be future targets in treating human diseases.

2. AIMS OF THE STUDY

As evident from the preceding chapters, c-Myb is an important player in the development and function of hematopoietic cells. Therefore understanding the molecular mechanisms governing c-Myb function and activity is highly important. Uncovering and characterizing the macro-molecular interactions and post-translational modifications in which c-Myb engages, will hopefully reveal new aspects of c-Myb biology. Moreover, knowledge about how c-Myb is regulated, might give a hint to how this proto-oncoprotein may be deregulated in hematopoietic cancers.

When this study was initiated a successful yeast two-hybrid screening had already been conducted in our lab, using full-length c-Myb as bait [81]. This screening identified five novel c-Myb-interacting proteins. One of these was Mi-2α, reported to be an integral component of the NuRD co-repressor complex [367], while the huge nuclear factor FLASH, reported to be a gene product essential for cell division [368, 369], was another. Furthermore, the SUMO E2 conjugating enzyme Ubc9 interacting with and promoting sumoylation of c-Myb was reported by our group in 2003 [81]. This had introduced us to the field of SUMO-biology. With new knowledge on the consequences of SUMO-conjugation, and lately non-covalent SUMO-binding, we were eager to get a better understanding of how this related to c-Myb function. Accordingly, the present work had two main objectives:

1. Characterize the interaction and function of the two novel c-Myb interacting proteins Mi-2α and FLASH. This included:
    (a) Substantiate and validate the interaction.
    (b) Examining whether c-Myb is part of protein-networks already described for the novel interaction partners.
    (c) Investigating the functional implications for c-Myb activity.
    (d) Trying to unravel the mechanisms underlying the putative implications in c.

2. Examine how c-Myb might be regulated by SUMO. This included:
    (a) Investigating whether c-Myb through SUMO-conjugation is subject to the phenomenon of synergy control, and how this might be escaped.
    (b) Exploring whether c-Myb is able to interact non-covalently with SUMO, and if so, what the functional consequences of this might be.

3. SUMMARY OF PAPERS

3.1 PAPER I

Sæther T, Berge T, Ledsaak M, Matre V, Alm-Kristiansen AH, Dahle Ø, Aubry F, Gabrielsen OS. (2007) The chromatin remodeling factor Mi-2α acts as a novel co-activator for human c-Myb. *J Biol Chem*, **282**, 13994-14005.

In the first paper we report the novel interaction between human c-Myb and the chromatin remodelling factor Mi-2α. We identified Mi-2α as an interaction partner for human c-Myb in a yeast two-hybrid screening where we used full-length c-Myb as bait. This screening was performed in a mixed cDNA library [81]. The main interacting domains in c-Myb and Mi-2α were mapped to the N-terminal region of Mi-2α and the DNA-binding domain of c-Myb using GST pull-down, with a weaker contact also detected between the FAETL domain of c-Myb and the C-terminal region of Mi-2α. The interaction was also confirmed with co-immunoprecipitation and in a more functional context by mammalian two-hybrid.

Mi-2α/CHD3 and the highly related Mi-2β/CHD4 belong to the CHD (chromo-helicase-DNA binding) protein family [370]. Mi-2α and -β are reported to be integral components of the NuRD (*nu*cleosome *r*emodelling and histone *d*eacetylase) co-repressor complex, responsible for its chromatin remodelling activity [367]. We were therefore surprised when functional analyses revealed that Mi-2α enhanced c-Myb dependent reporter activation. This was not due to any transient transfection artefact, since Mi-2α had no effect on the reporter gene expression when transfected alone and had no effect on the c-Myb expression or stability. Furthermore, a clear co-activating function of Mi-2α was also observed when monitoring the gene expression from the fully chromatinized target gene *mim-1* in HD11 cells. Consistently, knock-down of endogenous Mi-2α in c-Myb-expressing K562 cells led to down-regulation of the c-Myb target genes *NMU* and *ADA*. In addition, the Myb-Mi-2α transactivational co-operation was clearly potentiated by desumoylation of c-Myb, as well as co-transfection with p300.

Finally, when wild-type and helicase-dead Mi-2α were compared, the Myb-Mi-2α co-activation appeared to be independent of Mi-2α's ATPase/DNA helicase activity. The rationale for the unexpected co-activator function seems to lie in a dual function of Mi-2α, by which this factor is able to repress transcription in a helicase-dependent, and activate in a helicase-independent fashion, as revealed by Gal4 tethering experiments.

3.2 PAPER II

Alm-Kristiansen AH, Sæther T, Matre V, Gilfillan S, Dahle Ø, Gabrielsen OS. (2008) FLASH acts as a co-activator of the transcription factor c-Myb and localizes to active RNA polymerase II foci. *Oncogene*, accepted.

One of the other novel interaction partners for human c-Myb found in the yeast two-hybrid screening was FLASH. FLASH/CASP8AP2 was first identified as a component of the Fas-caspase-8 apoptotic pathway [368], but later reports have concluded that the main functions of FLASH are nuclear [371, 372]. Interestingly, a large siRNA screening in HeLa cells identified FLASH as one of 37 gene products essential for cell division [369].

We show that FLASH, like Mi-2α, interacts with the DNA-binding domain of c-Myb, and enhances c-Myb-dependent reporter activity as well as expression of endogenous c-Myb target genes. Interestingly, the FLASH-induced enhancement of c-Myb activity was comparable to the increase seen with the well-established c-Myb co-activator p300. Moreover, FLASH was demonstrated to bind AMV v-Myb, and to co-activate together with this leukemogenic version of c-Myb. siRNA knockdown of FLASH resulted in a reduction of the expression of the *MYC* and *ADA* genes, earlier shown to be *bona fide* c-Myb target genes [144, 204, 226]. This finding was strengthed by chromatin immunoprecipitation assay demonstrating that FLASH and c-Myb both associate with the *MYC* upstream promoter region, as well as with the intronic enhancer of *ADA*. Furthermore, Gal4 tethering assays revealed that the co-activator effect is mediated through the C-terminal part of FLASH, which both binds c-Myb and contains a functional activation domain.

Through immunofluorescence experiments we found FLASH to be localized in discrete nuclear speckles in several cell lines. Interestingly, these specles were highly co-localized with c-Myb in active RNA polymerase II foci. Altogether, these results suggest a novel molecular mechanism for regulation of c-Myb activity. We propose that c-Myb cooperates with FLASH in foci associated with active RNA polymerase II, leading to enhancement of Myb-dependent gene activation.

3.3 PAPER III

Molværsmyr AK, Sæther T, Lorenzo PI, Kvaløy H, Matre V, Gabrielsen OS. (2007) SUMO-conjugation of the transcription factor c-Myb controls cooperative behaviour and induces an activator-to-repressor switch in the negative regulatory domain. Manuscript.

We and others have earlier shown that c-Myb is sumoylated both by SUMO-1 [81, 82] and SUMO-2/3 [83] at lysines K503 and K527 in human c-Myb. Conjugation of both SUMO-isoforms to c-Myb lowers the transactivational potential of the transcription factor. Given the role of SUMO as a disruptor of synergy between other transcription factors [347, 350], we reasoned that studying its function in synergy control of c-Myb might lead to a better understanding of the mechanisms by which SUMO controls c-Myb action.

Systematic analysis of this type of behaviour required a dedicated set of reporter constructs with defined changes in the multiplicity of response elements. Hence we constructed a set of reporter plasmids with identical core promoters (from *MYC* P2), activated by one to five copies of an optimal Myb response elements (from *mim-1*). Using these reporters we were able to show that c-Myb is subject to a strong synergy control which is tightly linked to its level of SUMO-conjugation. In order to be able to quantify the difference in synergistic behaviour between different SUMO-related mutants of c-Myb, we defined a relative "synergy factor" (SF). By employing this factor we showed that wild type c-Myb in fact display "negative synergy" (SF~0.25) while the SUMO-negative c-Myb 2KR show a strong "positive synergy" (SF~4). Furthermore, synergy control is clearly abolished in v-Myb which produced the highest SF in this study (SF=4.8).

Based on the multiplicity of response elements and complexity of gene promoters, we expected the SUMO-governed synergy control to be promoter-specific. This was indeed the case, and we show that chromatin embedded target genes of c-Myb in hematopoietic cells respond differentially to SUMO-control. Finally, we show that SUMO affects the transactivation properties of the factor by switching off a heretofore unrecognized activation function in its negative regulatory domain. This contributes to the amplitude of activation and might explain the increase in synergy seen when removing the SUMO moieties by mutation or proteases. We propose a dual role for sumoylation of c-Myb in controlling both the transactivation properties and the synergy behaviour, ensuring a considerable dynamic regulation of c-Myb activity.

3.4 PAPER IV

Sæther T, Alm-Kristiansen AH, Troye Pettersen LK, Gabrielsen OS. (2007) A functional SUMO-binding motif in the transactivation domain of c-myb regulates its activity. Manuscript.

In the last paper we report a different kind of c-Myb-SUMO contact, namely the non-covalent binding of SUMO to c-Myb. We recently came to realize that loss of SUMO-conjugation sites is not the only oncogenic alteration in v-Myb that may relate to SUMO. Based on reports of a consensus SUMO-binding motif (SBM; aaa-V/I-V/I-X-V/I/L and V/I-X-V/I-V/I-aaa) [273, 274, 276], we analysed the c-Myb sequence and found that it contains two putative SBMs; one in the R2 repeat in the DNA-binding domain (termed SBM1), and one in the N-terminal end of the transactivation domain (termed SBM2). Remarkably, both sites are mutated in v-Myb.

GST pull-down and effector-reporter assays showed that SBM2 in the transactivation domain of c-Myb (V_{267}NIV) is functional. This motif can interact non-covalently with SUMO, and preferentially bind SUMO-2/3. Furthermore, when we mutated this motif and destroyed the SUMO-binding properties, a large increase in c-Myb transactivational activity was observed. Through different functional assays we show that this increase in activity was not due to impaired SUMO-conjugation, lost synergy control or intramolecular interactions being broken, but rather due to lost interactions in *trans*. Using confocal imaging of PML and wild-type or SUMO-binding mutants of c-Myb we show that c-Myb is recruited to PML nuclear bodies (PML-NBs) in a SUMO contact-independent manner. The c-Myb SBM mutant (A_{267}NAA), as well as the sumoylation-negative mutant (2KR) and the double mutant (ANAA 2KR), associates with the PML-NBs to the same extent as does c-Myb wild-type. Interestingly, the SUMO-independent recruitment of c-Myb to PML-NBs did not correlate with functional data showing that c-Myb wild-type is activated by ectopic PML expression, while the SBM mutant and the sumoylation-negative mutant has lost some of this potential. Moreover, the double ANAA 2KR mutant is close to unresponsive with regard to PML-dependent activation.

Based on these findings we hypothesize that c-Myb can be relived from negatively acting sumoylated and SUMO-binding factors through their sequestering in PML-NBs, and that this loss of co-repression is mimicked by the SUMO contact-negative mutations.

4. DISCUSSION

Transcription factor activity may be regulated in many ways; through dosage, i.e. through modulating the expression of the transcription factor, through posttranslational modifications of the factor, or through interactions with other proteins. This thesis has focused on two such mechanisms that regulate the activity of the proto-oncoprotein c-Myb; interaction with co-factors and posttranslational modification. Protein interactions and posttranslational modifications are often interlinked processes, and PAPER IV in the thesis may actually serve as an example of this. Here, a small motif able to bind the small ubiquitin-related modifier SUMO is identified in c-Myb. Nevertheless, I have chosen first to discuss the co-factor link, where the two novel c-Myb-interacting proteins Mi-2α and FLASH will be treated. Thereafter, I will shift focus to c-Myb and SUMO, where the mechanisms SUMO-conjugation, synergy control and SUMO-binding will be compared and discussed.

4.1 CO-ACTIVATORS OF c-MYB – PROCESSES AND PLAYERS

Several proteins have been reported to interact with different subdomains of c-Myb and influence its activity (see introduction; Tab 1). For some of these proteins the mechanism of co-regulation has been studied in great detail, C/EBPβ and CBP/p300 being two examples. Yet for others, the mechanisms are still not clear. In PAPER I and II in this thesis we report Mi-2α or CHD3, belonging to the chromo-helicase-DNA binding protein family, and FLASH, a gene product essential for cell division, as novel interacting partners for c-Myb. Both of these proteins function as co-activators in a c-Myb context. Through functional assays we have partly uncovered the mechanisms responsible for these effects. However, many questions still remain to be answered.

The co-activator principle was first coined in 1990 by Pugh and Tjian, working on the transcription factor Sp1 in reconstitution reactions [373]. Sp1 was shown to be unable to stimulate transcription at TATA-containing promoters, using purified, cloned Drosophila or yeast TFIID. However, in the presence of semi-purified TFIID fractions from either human or Drosophila cells, this became possible [373], indicating that some unknown proteins were able to function as molecular adaptors between transcription factors and the general transcription initiation machinery. With the understanding of non-DNA-binding nuclear proteins still being both necessary and able to activate transcription, a second dimension

was added to the field of transcription. Today, co-activators are thought to exert their function either through bridging of upstream transcription factors and the pre-initiation complex, or through the recruitment of chromatin remodelling or modification enzymes. In general these two mechanisms of actions might not be so different, depending on whether one includes indirect interactions or not. Many co-activators either have intrinsic chromatin remodelling or modification activity, or they recruit such enzymes to chromatin. Hence, modulation of chromatin dynamics is recognized as a fundamental way to regulate gene expression [12-14].

4.1.1 Remodelling the remodeller - from NuRD to PHD

When we identified Mi-2α as a c-Myb interacting protein (PAPER I) we expected it to exert its function as part of the NuRD (*Nu*cleosome *r*emodelling and histone *d*eacetylase) complex, which possesses both chromatin remodelling and histone deacetylase activity and is involved in repression of gene expression [367]. Still, we found it somewhat puzzling that the transcriptional activator c-Myb might be involved in gene repression. Nevertheless, c-Myb has been hypothesized to participate in establishing chromatin patterns specific for hematopoietic genes, due to its role as an early hematopoietic transcription factor [374]. This could potentially be achieved through promotion of a restricted chromatin state, leading to gene silencing. Yet, when we co-transfected c-Myb and Mi-2α, we saw the opposite. Mi-2α had a co-activating effect on Myb-dependent transactivation (PAPER I). These data were verified on several reporters and shown to be valid also for the fully chromatinized c-Myb target gene *mim-1*. The simplest explanation for this would be that overexpression of Mi-2α created a dominant negative effect, by titrating out other components of the NuRD complex. However, we were able to show under more physiological conditions, that knock-down of endogenous *CHD3* (Mi-2α) in c-Myb expressing K562 cells down-regulated the expression of the *bona fide* c-Myb target genes *NMU* and *ADA* [196, 204, 226]. Removing the factor that potentially might be targeting the NuRD co-repressor complex to c-Myb actually lowered the expression from these promoters. In line with this, co-immunoprecipitation experiments using antibodies to precipitate NuRD subunits such as HDAC1, RbAp46 and MTA2 both in transfected cells and with endogenous factors only detected very weak interactions with c-Myb.

The evidences regarding the composition of the NuRD complex are conflicting [367]. This probably reflects its heterogeneity as well as its tissue and target specificity. The

consensus NuRD complex consists of HDAC1 and -2, RbAp46 and -48, Mi-2α and/or -β [375-377], one or more MTA proteins (MTA1, -2 or -3, or splice variants of these) [378], MBD2 or MBD3 [379], and often p66α and/or -β [380]. Furthermore, the finding of a tripartite HEB:Mi-2β:p300 complex that is able to bind to the *CD4* enhancer region in T cells, increase histone H3 acetylation and enhance *CD4* gene transcription [381], implies that the helicase partners in the NuRD complex might be involved in gene activation. p300/CBP is a well established c-Myb co-activator [78, 79, 382], and we could show that p300 potentiated the Myb-Mi-2α transactivational co-operation. Nevertheless, we were not able to detect any physical interaction between p300 and Mi-2α or c-Myb:Mi-2α. Hence we were left with the possibility that the co-activation function of Mi-2α was coupled to its helicase activity.

To our big surprise, destroying the ATPase/helicase activity by mutations did not eliminate Mi-2α's ability to co-activate together with c-Myb. However, when fused to Gal4p DBD and tethered directly to two Gal4p-responsive promoters, only the helicase-dead mutant activated the reporter. The wild-type on the other hand repressed the reporter. Finally, we were able to show that the activation domain of Mi-2α seemed to reside within its N-terminal domain, the same domain binding to c-Myb. Thus, Mi-2α seems to harbour a dual function, where it is able repress transcription in a helicase-dependent and activate in a helicase-independent fashion.

The functional role of the helicase activity of Mi-2β in the HEB:Mi-2β:p300 complex was not addressed by Williams and co-workers in their work on Mi-2β-dependent activation of *CD4* gene transcription [381]. It is, however, possible that also Mi-2β, which is highly homologues to Mi-2α, activate the transcription of *CD4* in a helicase-independent manner. Still, these are only speculations. Independently, in a c-Myb context Mi-2α may function as a classical co-activator by bridging c-Myb to other transcription-related factors. Which protein(s) this might be is still an open question, but the link to histone H3 is intriguing (Fig 17A). In 2005, the Leutz group demonstrated a SANT function for c-Myb [54]. The DBD/SANT of c-Myb binds to the N-terminal tail of histone H3 between amino acid residues 27-42 and facilitates acetylation of H3 lysines. This leads to gene activation of *mim-1* in HD11 cells. [54]. Mi-2α is also able to bind histone H3, and has the properties of a histone-code reader [383]. Particularly interesting is the fact that the Mi-2α PHD (Plant homeodomain) associates with exactly the same tail-region of histone H3 as the DBD of c-Myb. Moreover, as shown in PAPER I, it is the N-terminal region of Mi-2α, adjacent to the

PHD fingers, that interacts with c-Myb DBD. Hence, Mi-2α and c-Myb might meet on chromatin, thus strengthening the concept of c-Myb as a SANT factor (Fig 17A).

To date, no modification-specific histone-binding has been observed for c-Myb SANT. In contrast, the first of Mi-2α's two PHD fingers, residing in the N-terminal region, show specific binding to H3 K36me3 [383]. Methylated H3K36 is found enriched in active chromatin where K36me3 generally accumulates toward the 3′ region of transcribed genes [384]. Theoretically, the Mi-2α PHD might augment the specificity of the c-Myb histone-binding: By binding to c-Myb, Mi-2α may bridge this transcription factor to a specific activation mark on histones. Hence it would be interesting to study how c-Myb and Mi-2α cooperate at the chromatin level. Several questions seem relevant: Is Mi-2α stabilizing or destabilizing the c-Myb:histone interaction? Is Mi-2α able to bind both c-Myb and histone H3 at the same time, or is Mi-2α bridging c-Myb with adjacent histones? Is c-Myb capable of binding histone H3 and DNA at the same time or are these mutually exclusive events? And finally, do the interactions outlined in Figure 17 happen simultaneously or sequentially? This last question might be highly relevant, since more and more histone modifications have

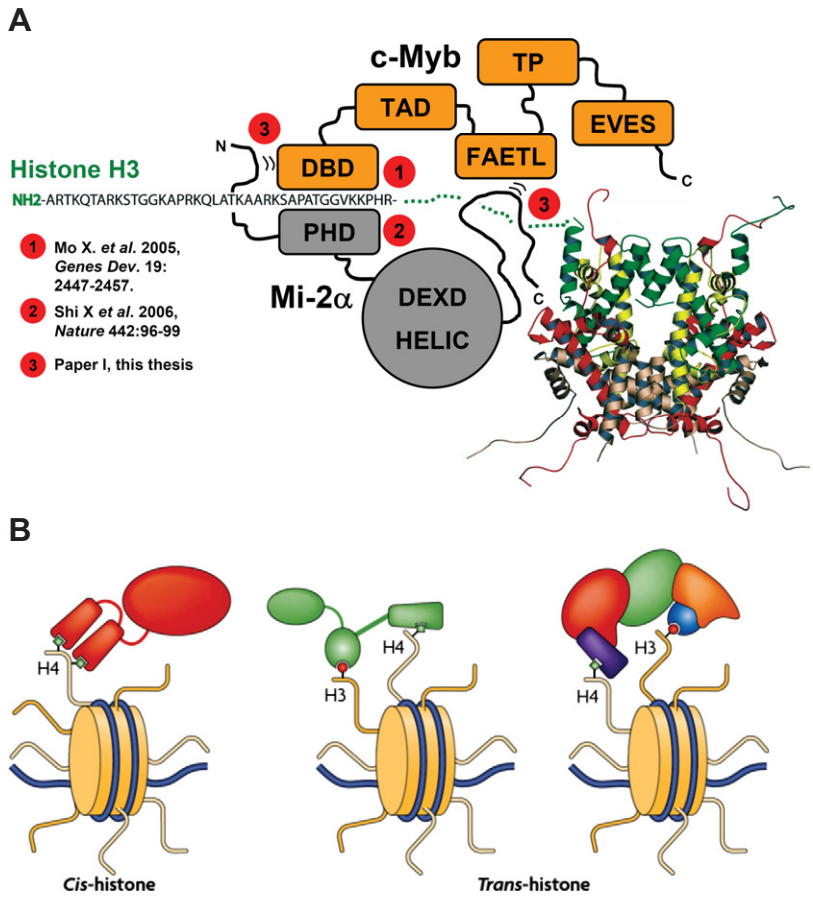

Figure 17 A Myb-meeting for the future. *A* Mi-2α and c-Myb both binds histone H3 in the same region of the N-terminal tail (amino acid residues 27-42). At the same time the N-terminal domain of Mi-2α interacts with c-Myb DBD. A second, much weaker interaction is seen between the C-terminal region of Mi-2α and c-Myb. The numbered red dots refer to the papers within which these interactions have been described. *B* Cooperative binding of PTMs on a single (*cis*) or two different (*trans*) histone tails. When more than one discrete binding contact is made the specificity is thought to increase. Most of these crucial interactions are envisioned as modification-dependent. Modified from illustration in [385]

been shown to occur in a highly ordered fashion, where one promote the next [386, 387]. Furthermore, as evident from studies of several histone-code reading modules like PHD, Bromo, Chromo and Tudor, a given combination of modifications might facilitate binding of one factor and at the same time exclude others (reviewed in [385]; Fig 17B).

The histone-code reader functions of Mi-2α might be essential for co-activation of c-Myb. If so, simultaneous binding of DBD and histones could bring c-Myb in contact with more distal genomic elements. Through Mi-2α-facilitated looping of chromatin, c-Myb might be positioned in the vicinity of other transcription factors which otherwise would be beyond reach, favouring synergistic gene activation. In this context it is interestingly to notice that Mi-2α seemed to activate better on natural promoters *TRHR*, *RAG-2* and *mim-1* (PAPER I) than on synthetic promoters with multiple, 10 bp-spaced MREs (data not shown). This may indicate that when an array of c-Myb proteins is binding to the promoter, the 240 kDa Mi-2α is prevented from interacting with DBD. Alternatively, it may allude to the fact that Mi-2α mainly plays a role in bridging c-Myb with other (more distal) factors, and that this potential already is unleashed when multiple c-Myb-species are bond at close proximity.

In view of c-Myb as one of two piers in a transcriptional bridge, where Mi-2α is the bridge section, a study on Mi-2β might give a hint towards another pier candidate. Shimono and co-workers have shown that BRG1, a SWI/SNF component, interacts with the N-terminal domain of Mi-2β in a co-activating fashion [388]. This corresponds to the domain in Mi-2α that shows the highest affinity for c-Myb, and in fact, this domain alone was enough to enhance the dependent reporter activation to a level comparable to that of full-length Mi-2α (PAPER I). All in all, the results from our study, combined with previous reports on the co-activating functions of Mi-2β, suggest that the concept of Mi-2 proteins being only the helicase components of a single repressive NuRD complex is too simplistic.

4.1.2 Activated by a FLASH

The second novel interaction partner of c-Myb described in this thesis is FLASH (FLICE associated huge; PAPER II) also known as CASP8AP2 (caspase 8 associated protein 2). In contrast to Mi-2α and c-Myb which both are known to be nuclear factors, FLASH was originally described as being cytoplasmic [368]. This suggested that the interaction between FLASH and c-Myb might be an artefact stemming from the yeast two-hybrid screening, where proteins of all subcellular localization are allowed to meet in the nucleus. In the first reports on this protein, FLASH was shown to interact with procaspase-8 and claimed to be

(but not shown to be) located mainly in the cytoplasm [368]. However, in another paper endogenous FLASH was found to be able to shuttle to the nucleus upon TNFα-stimulation [389]. The FLASH protein harbours two putative nuclear localization signals (NLS) and one putative nuclear export signal [368]. Hence, the subcellular localization of this protein was not obvious. Therefore, we carefully studied the distribution of FLASH using both imaging techniques and subcellular fractionation in combination with western blotting. In all our experiments, we found FLASH to be localized in nucleus. Since the same patterns were seen when using antibodies against both the N- and C-terminal parts of FLASH, the entire protein must be found in the nucleus. Finally, during this work two other groups published evidence supporting a nuclear function for FLASH [371, 372], allowing us to continue the work on the c-Myb-FLASH connection.

Like Mi-2α, FLASH co-activates together with c-Myb. This was shown using transient transfections in combination with effector-reporter assays as well as by monitoring the expression of the resident *mim-1* gene in the chicken macrophage cell line HD11. Furthermore, knockdown of endogenous *FLASH* in K562 cells resulted in down-regulated *MYC* and *ADA* gene expression, previously shown to be responding the same way by *MYB* knock-down [226]. Moreover, through chromatin immunoprecipitation assay, we showed that both c-Myb and FLASH were associated with the *MYC* upstream promoter region as well as the intronic enhancer of *ADA*. Using Gal4 tethering assays we located a transactivation function in the C-terminal region of the protein, the same domain that interacts with c-Myb. Similar to Mi-2α, FLASH interacts with the DNA-binding domain of c-Myb (PAPER II). Interestingly, FLASH seems to harbour a SANT-like domain in this part of the protein. If functional, the SANT domain may further link FLASH to chromatin and nuclear functions.

Using immunofluorescence and confocal laser scanning microscopy we observed that both transfected and endogenous FLASH localized in nuclear speckles in several cell lines, with only a weak diffuse distribution in the nucleoplasm and cytoplasm (Fig 18; PAPER II). c-Myb was found to be co-localized with these foci, and upon co-transfection with FLASH observed in punctual structures in close to 100% of the c-Myb positive cells. The FLASH foci were demonstrated to be partly co-localized with PML nuclear bodies (PML-NBs; described in chapter 1.3.2.3) and Cajal bodies (CB). The CBs have been implicated in replication-dependent histone gene transcription and mRNA maturation, and assembly of the three eukaryotic RNA polymerases (pol I, pol II, and pol III) with their respective transcription and processing factors [371]. However, since FLASH only partly

localized to these structures, we believe that the FLASH proteins form distinct "FLASH bodies" located in close association with both PML-NBs and CBs (PAPER II).

Interestingly, the nuclear protein that FLASH most often seemed to be co-localized with is active RNA polymerase II. Using antibodies towards RNA pol II, phosphorylated at Ser5 in the carboxy-terminal domain, we were able to show that both c-Myb and FLASH co-localized to the same active RNA polymerase II foci. These results imply a novel molecular mechanism of regulation of c-Myb activity. When adapting these data to a classical co-activator view, FLASH might be a factor able to bridge c-Myb with the general transcription machinery. As evident from PAPER II, we do not have any evidence for a direct c-Myb-FLASH-RNA pol II interaction. However, we propose that c-Myb cooperates with FLASH in foci associated with active RNA polymerase II, leading to enhancement of Myb-dependent gene activation.

4.1.3 A friend in common does not imply contact

Transcription factors typically exert their function in larger protein complexes. However, with a few exceptions, multimeric complexes have so far not been described in greater detail for c-Myb. Having reported two novel co-activators for c-Myb, both binding to c-Myb DBD, we were inspired to do some simple follow-up experiments aiming at disclosing any potential of mutual cooperation. Furthermore, since c-Myb and Mi-2α have, and FLASH might have an inherent affinity for histones, this could potentially strengthen the c-Myb chromatin link. Given a tripartite cooperation on c-Myb-responsive promoters, we expected no less than an additive effect on the reporter output when the three factors where co-transfected. However, when calculating the fold-contribution of e.g. FLASH to the reporter activation in c-Myb/Mi-2α/FLASH-transfected cells, this was exactly what we got (data not shown). Hence, Mi-2α and FLASH do not seem to potentiate each other in Myb-dependent gene expression.

When examining the data form PAPER I and II this might be plausible. After all, knock-down of *CHD3* (Mi-2α) had an effect on *ADA* and *NMU*, but not *MYC*, while *FLASH* knock-down affected *MYC* and *ADA*, but not *NMU.* It is obvious that the c-Myb co-activators identified in these studies have some kind of target gene preference, either autonomously or when bound to c-Myb. Target gene specificity based on posttranslational modification and repertoire of interacting co-activators or promoter-bound sequence-specific transcription factors, has been discussed for c-Myb [390]. Moreover, given the

latent promiscuity of c-Myb:DNA interaction this would be an obvious level to increase specificity.

The nuclear distribution of the three proteins in question also indicate that c-Myb, FLASH and Mi-2α at best only physically interact in a small fraction of cellular time-space (Fig 18). Interestingly, the nuclear staining of c-Myb represents a combination of punctual structures and even distribution, resembling the pattern of FLASH and Mi-2α, respectively. FLASH and c-Myb co-localize in the FLASH-foci (PAPER II), while the co-localization of c-Myb and Mi-2α is more difficult deduce, due to the even distribution of Mi-2α (PAPER I). Still, the bipartite nuclear pattern of c-Myb indicates multiple roles for this protein, which should allow for both FLASH and Mi-2α interaction.

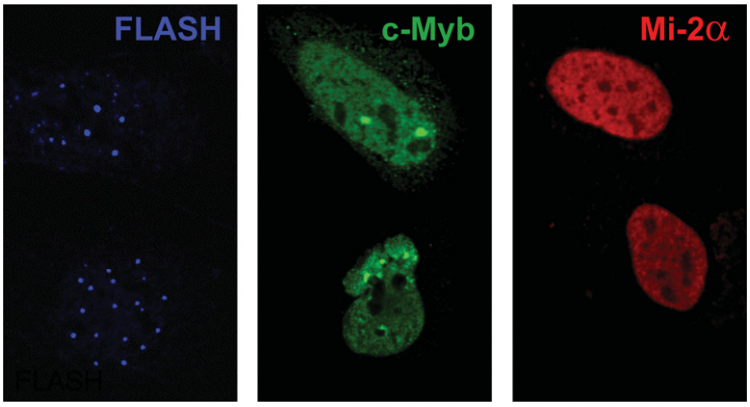

Figure 18 A friend in common does not imply contact. Cellular staining of FLASH (blue), c-Myb (green) and Mi-2α (red). The cells depicted show a distribution pattern representative for the three proteins. The panels display six different cells, all transfected with equal amounts of the relevant expression plasmids.

FLASH and Mi-2α have a diverging nuclear distribution. Nevertheless, only ChIP or better re-ChIP assays on multiple c-Myb target gene promoters will determine whether FLASH and Mi-2α co-interact with c-Myb in Myb-dependent transcription. This also applies to other known c-Myb co-factors or cooperating transcription factors. We show in PAPER I that Mi-2α enhances c-Myb-p300 transactivational activity. Still, we were not able to detect any direct or indirect physical contact between p300 and Mi-2α. Furthermore, the co-activator ability of FLASH resembles p300 (PAPER II), but their co-activation and interaction was not addressed. It is however possible that p300 and Mi-2α or FLASH cooperate on c-Myb responsive promoters without physically interacting. Other proteins worth considering in relation to FLASH and Mi-2α co-activation of c-Myb are factors

reported to interact with c-Myb DBD, e.g. p100 and C/EBPβ. A large number of proteins have been reported to bind to this part of c-Myb, hence it would be interesting to examine whether some of these interactions are excluded or facilitated by FLASH and/or Mi-2α. Since both FLASH and Mi-2α interacts with c-Myb DBD and co-activates transcription, DBD-binding co-repressors like c-Maf, MafB, RARα, Cyclin D1/D2, c-Ski and Cyp40 would be candidates for mutually exclusive interacting proteins (Table 1; chapter 1.2.3.1). For the reported co-activators/transcription factors interacting with DBD several options are conceivable.

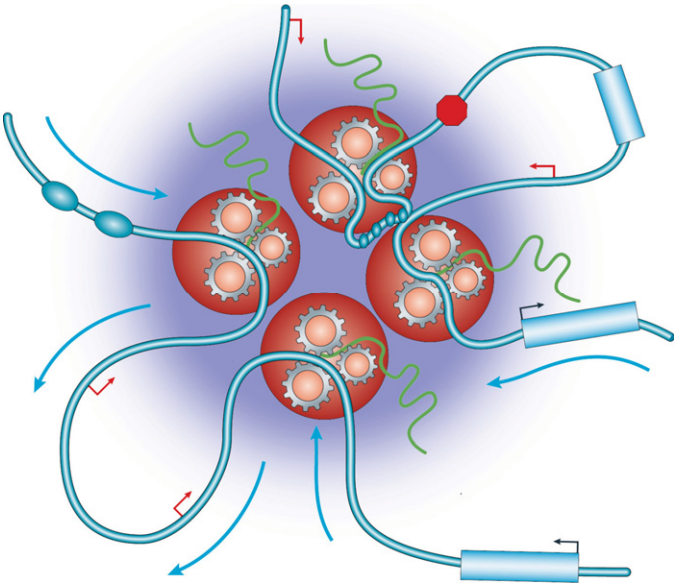

Figure 19 The FLASH-body - a transcription factory? In this model, genes extend out of their "chromosome territories" to access a shared transcription factory. Clustered RNA polymerase II motor proteins (red spheres ^{w}/cog wheels) organize genes and distal regulatory elements. Rather than the polymerase sliding along chromatin, chromatin moves through the factory that is powered by the energy released during synthesis of RNA (green). Could FLASH (blue halo) be a part of such factories? Modified from image in [391].

4.1.4 The FLASH-body - a transcription factory?

An intriguing aspect of our report on FLASH and c-Myb is the set of data showing that both c-Myb and FLASH co-localize to active RNA polymerase II foci (PAPER II). Moreover, when counting FLASH-positive foci as much as 75 % of these were co-localized with active RNA polymerase II. This suggests that these subnuclear bodies are sites of active transcription where all three proteins operate together to enhance gene activation. In the last years, the links between active transcription and nuclear structure have been strengthened [391]. When looking at active RNA polymerase II foci in the cell, the number of these can not account for the number of genes being transcribed. This has led to the prediction that more than one active gene is transcribed in each RNA pol II foci, which have been termed **transcription factories** [392, 393]. Furthermore, recent results show that actively transcribed genes that are separated by up to 40 Mb of chromosomal sequence frequently co-localize in the same transcription factory (reviewed in [391]). Such a model would also

explain the fact that locus control regions or distal enhancers located several kb away from the transcription start site still regulate transcription and engage in specific higher-order, 'loop' structures. The question then becomes if FLASH is part of these factories, and furthermore which role it plays in recruiting sequence-specific transcription factors to these nuclear units. With the finding of FLASH in CBs, the latter being implicated in assembly of the RNA polymerases [371], and our finding of a highly frequent co-localization between FLASH and active RNA pol II (PAPER II), we think that the transcription factory model may become particular relevant for future FLASH research. With the novel finding of c-Myb target genes residing in close proximity to origins of replication [185, 186, 189], it is interesting to notice that the transcription factory model also embrace replication and replication factories [391]. Despite being separate entities, relocation to or from transcription or replication factories would favour early replication and transcription in the same chromosomal area.

4.1.5 Essential gene products and co-activation of cancer

Although it has been known for some time that c-Myb is a gene product essential for the hematopoiesis [160, 164] and that AMV and E26 v-Myb cause leukemia in chicken (reviewed in [16]), the links to human cancers of the blood was for a long time obscure. However, the identification of recurrent genomic aberrations involving the *MYB* locus in T cell acute lymphoblastic leukemia (T-ALL) emphasized that c-Myb may play a role in the development of human leukemic disorders [242-244]. A common theme in both chicken and human leukemias involving c-*myb*/*MYB* anomalies is that the overall Myb-activity seems to be increased: **I:** The v-Myb proteins are stronger activators than c-Myb when tested on standard Myb-responsive reporters [16, 42]. **II:** In T-ALL the translocation, t(6;7)(q23;q32~36) places *MYB* in the vicinity of the T cell receptor B promoter, leading to significantly higher expressional levels of *MYB* than in other T-ALL patients [243], while **III:** local duplication of the *MYB* gene has the same effect [243, 244]. According to AM. Gewirtz, most human leukemias analyzed in his lab at the University of Pennsylvania show over-expression of c-Myb (AM. Gewirtz, 4th MYB workshop, Italy, 2007)

Given that increased c-Myb levels, hence activity, might be involved in the transformation of hematopoietic cells, one may expect that other perturbations resulting in increased Myb action will have the same effect. When reasoning along these lines, one type of candidate involved in inducing c-Myb transactivational activity is co-factors. Interestingly, both co-factors reported in this thesis have been indirectly linked to human

neoplasias. Mi-2α was first identified as an autoantigen in the human connective tissue disease, dermatomyositis [394, 395]. The underlying mechanism for this is not known. Still several clinical studies have linked dermatomyositis as an external marker and an early manifestation of different forms of leukemia [396-399].

FLASH has been found to be a promising prognostic marker in childhood acute lymphoblastic leukemia (ALL; [400]). In a cohort of 99 patients, low levels of *FLASH* expression predicted a lower event-free survival after chemotherapy and a higher rate of leukemia relapse, while high levels were associated with a greater tendency of leukemic lymphoblasts to undergo apoptosis. As mentioned earlier, FLASH was originally thought to be exclusively cytoplasmic [368]. However recent research (ours included) has shown that FLASH indeed has a nuclear function [371, 372, 401]. Nevertheless, FLASH can shuttle to the cytoplasm upon the activation of the death receptor CD95 [372]. Here it associates with mitochondrial caspase-8 and promote apoptosis. Regarding the clinical trial where high levels of *FLASH* were shown to be beneficial for chemotherapy treatment in an apoptosis-dependent manner, it is most likely that this is linked to the cytoplasmic functions of FLASH. Recently, *FLASH* was found to be mutated in gastric adenocarcinomas [402]. Earlier, *FLASH* has also found to be mutated in DNA mismatch repair (MMR) defective colorectal cancer cell lines [403]. This was however not correlated to any biological feature of these cell lines. Thus, it is still an open question as to how the FLASH mutations contribute to the pathogenesis of these neoplasms. With the risk of overrating the significance of these reports, it is intriguing that FLASH function and/or deregulation has been linked to cancers of both blood and colon – two tissues in which c-Myb aberrations have been shown to be associated with cancer [8, 132, 133].

Finally, FLASH has also been identified as one of 37 gene product essential for cell division, based on the observation that FLASH mRNA knockdown caused the cells to die upon entry into mitosis [369]. These observations seem to contradict the notion that FLASH is involved in promoting apoptosis. However, this might once again highlight the multiple functions of this protein. Through its association with the Cajal bodies FLASH is directly involved in cell cycle maintenance [371]. The Cajal bodies have been shown to be essential for S-phase progression due to their close association with histone gene clusters, and FLASH have been demonstrated to positively regulate the expression of histone genes [401]. Hence, it is likely that it is the nuclear functions of FLASH that are indispensable for cell division. Even though c-Myb does not seem to be involved in the transcription of replication-dependent histone genes, as evaluated by *MYB* knock-down (EM. Brendeford,

unpublished results), it would be interesting to assess whether FLASH affects the activation of other histone genes, like *H2A.Z* reported to be a c-Myb target [199].

4.2 SUMO CONJUGATION AND BINDING - INTERPLAY OR TWO PROCESSES?

Through the last decade, studies within the field of SUMO (Small Ubiquitin-like modifier) have continuously revealed new and fascinating roles for this small nuclear peptide, and the number of targeted proteins is constantly growing [404]. We and others have shown that c-Myb is sumoylated both by SUMO-1 [81, 82] and SUMO-2/3 [83] at lysines K503 and K527 (human c-Myb numbering). The conjugation of both SUMO-isoforms to c-Myb lowers the transactivational potential of the transcription factor [81-83]. Even though sumoylation of c-Myb is established, the mechanistic explanation of how SUMO controls c-Myb action is still lacking. Given the role of SUMO as a disruptor of synergy between other transcription factors [347, 350], we reasoned that examining whether this function also operates on c-Myb might lead to a better understanding of SUMO and sumoylation in c-Myb biology (PAPER III).

In addition to suppressing the activity or synergistic potential of transcription factors, covalent conjugation of SUMO has been shown to alter the localization and interaction repertoire, or increase the stability of nuclear factors [253, 254, 258, 259]. The common assumption has for a long time been that most effects of SUMO have to be mediated through protein interactions. Therefore, the recent advances with regard to non-covalent binding of SUMO, and the identification of protein motifs responsible for such interactions has reinforced the framework within which SUMO-biology is interpreted (reviewed in [261]). This led us to address whether non-covalent SUMO binding also is an intrinsic function in c-Myb (PAPER IV).

4.2.1 SUMO-conjugation of c-Myb: wrestling with synergy

In PAPER III we show that c-Myb is indeed subject to strong SUMO-governed synergy control (SC). Systematic analysis of this type of behaviour required a dedicated set of reporter constructs with defined changes in the multiplicity of response elements. Using such reporters we were able to show that c-Myb wild-type activated the different reporters only with minor differences, while the SUMO-conjugation deficient 2KR mutant displayed a dramatic increase in activation with increasing amounts of MREs (PAPER III; Fig 22). Calculating the synergy factor ($SF=RLU_{4\times MRE}/4\times RLU_{1\times MRE}$) showed that while c-Myb wild-type displayed “negative synergy” with an SF = 0.26, c-Myb 2KR showed strong

"positive synergy" with an SF = 3.9. Further experiments using a battery of mutants interfering with sumoylation of c-Myb in different ways and overexpression of the SUMO-protease, SENP1, allowed us to conclude that the SC of c-Myb is highly dependent on the sumoylation status.

Synergy between transcription factors operating together on complex promoters is a well-known phenomenon. However, the link between control of the synergistic properties of a transcription factor and its ability to be sumoylated, was first observed in studies of the glucocorticoid receptor (GR; [347, 348]). Later this was extended to other factors such as C/EBPα, SF-1 and MITF [350, 405, 406]. As for c-Myb, the SC properties of all these transcription factors were studied on specially designed reporters harbouring multiple factor-specific response elements (REs). It is, however, obvious that only a tiny fraction of natural promoters has such a set-up, with evenly spaced and identical REs. Thus for these findings to be relevant, SC must be a mechanism that is operating also on promoters responding to several different factors, given that some of them are sumoylated. Consequently, promoters that are not subject to SC probably also exist, due to the fact that they only respond to transcription factors that cannot be sumoylated, or because of alleviative distances in time or space between the activators or REs. We therefore reasoned that the fold-difference in activation of resident promoters between c-Myb wild-type and 2KR ought to differ from gene to gene. When comparing the activation of the *mim*-1, *lysozyme* and *pdcd4* gene in chicken HD11 cells we indeed observed a difference in SUMO-controlled gene expression. For the *mim*-1 gene there was a 3.3-fold difference in activation between 2KR and wild-type, while the same ratio for the *lysozyme* and *pdcd4* genes were 1.2 and ~1, respectively.

The *mim*-1 gene is co-activated by c-Myb and members of the C/EBP family, and these have been shown to synergize [97]. More specifically, v-Myb134 (a v-Myb version with c-Myb back-mutations in DBD) and a C-terminally truncated c-Myb were able to synergize with C/EBPβ in activating the *mim*-1 gene, while c-Myb wild-type was not [97]. Interestingly, we now know that neither v-Myb134 nor the C-terminally truncated c-Myb is sumoylated, indicating that the ability of C/EBPβ and c-Myb to synergize on the *mim*-1 promoter probably is highly SUMO-dependent. Even more interesting are observations from the same study showing that C/EBPβ and v-Myb134 are unable to synergize on the *lysozyme* promoter [97]. Our data echoes this finding, and indicates that the lack of synergistic behaviour on the *lysozyme* promoter probably stems from spatial and/or temporal

separation of c-Myb and C/EBPβ and not from SUMO-governed synergy control. C/EBPβ as well as other members of the C/EBP family are covalently modified by SUMO [407]. Hence, also the sumoylation status of C/EBPβ might influence the synergistic output from the genes studied in PAPER III. Therefore it would be interesting to study the regulation of the same genes when co-expressing SUMO-negative C/EBPβ and c-Myb, alternatively when overexpressing SENP1.

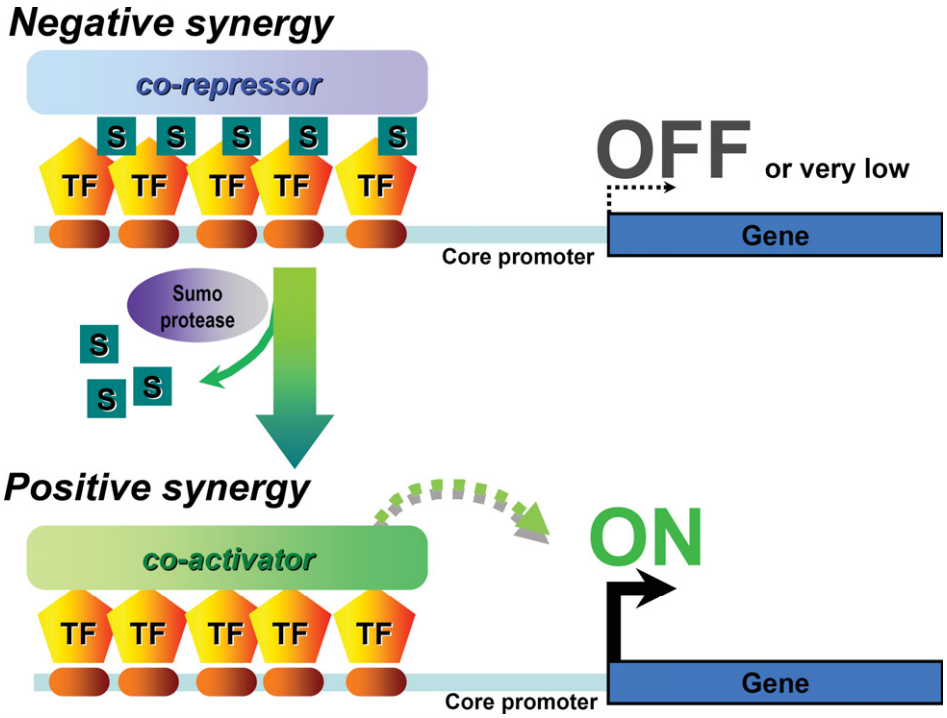

Figure 20 A model for a SUMO-dependent promoter-switch. SUMO-conjugation of multiple transcription factors create an array of closely spaced SUMO-molecules that either recruits negatively acting co-regulators or interferes with positively acting co-activators, keeping the promoter more or less off (negative synergy). Removing this array allows the transcription factors to recruit positively acting co-regulators, and the promoter to be turned on to high levels (positive synergy). In this model removal of SUMO-conjugation is a prerequisite for activation of complex promoters. One way of achieving this would be through controlled recruitment of SUMO-proteases. TF: transcription factors, S: SUMO.

In previous papers on SUMO-governed SC, most of the focus has been on how SUMO might effectuate this control [347, 350]. The idea has been that the SC can be achieved through the recruitment of negatively acting factors, or so-called synergy control factors (SCF) via multivalent binding to SUMO-moieties on adjacently bound regulators [350]. In the light of the recent identification of motifs for non-covalent SUMO-binding ([273, 274, 276]; see chapter 1.3.3) this theory seems plausible. Moreover, several factors known to repress transcription seem to harbour multiple putative SUMO-binding motifs.

One example here are the HDACs, where HDAC6 has the highest number (=5). Importantly, we did not see any increase in synergy when treating the cells with the HDAC inhibitor trichostatin A (PAPER III), suggesting that the SC seen with c-Myb wild-type is not dependent on HDAC enzymatic activity. Furthermore, the existence of an SCF at best explains the negative synergy seen with sumoylated transcription factors (PAPER III). Potential SCFs will not account for the flipside of synergy control, namely positive synergy. Several models have been proposed to explain the more-than-additive activity of multiple activators, and these may still be applicable even when a layer of SUMO-control is added. Some of the key concepts suggested have been multiplicity of contacts to the basal transcription apparatus [408, 409] promoting assembly of the pre-initiation complex [410], physical interactions between transcription factors [411, 412], and co-activators harbouring distinct domains that interact simultaneously with different factors [413]. When trying to explain both positive and negative synergy, multiplicity is an important aspect. One way of incorporating this is to assume the recruitment of a multivalent co-repressor (e.g. an SCF), able to interact with arrays of SUMO-conjugated factors, and multivalent recruitment of co-activators to the non-sumoylated array.

Even thought the concept of synergy assumes cooperativity, it is however important to emphasize that this not necessarily implies concurrency, i.e. simultaneous contact between a given co-regulator and all the transcription factors in the promoter array. Using c-Myb as an example, one might take into consideration that when increasing the number of promoter-bound species from one to two or three, this will double or triple the concentration of a given c-Myb interaction surface at the promoter, respectively. Thermodynamically however, this will result in a more than additive increase in activator-co-factor complex formation, although the co-factor might be bound to only one c-Myb species at a time. Nevertheless, it is interesting to notice that when changing the helical orientation of the bound Myb-species by increasing the MRE-spacing to 15 bp, the level of synergy control becomes more relaxed, while the level of positive synergy remains unaltered (PAPER III). This may reflect that different mechanisms of cooperation are responsible for restricting as compared to promoting synergy. More precisely, a synergy control factor might be more dependent on multiple SUMO-contacts for efficient binding than a synergy promoting factor is on concurrent binding of multiple TADs.

As seen in Figure 20 we propose a model where SUMO-modified transcription factors create an array of SUMO-molecules that either recruits co-repressors or interferes with the recruitment of co-activators, keeping the promoter more or less off (negative

synergy). Removing this array allows the transcription factors to recruit positively acting co-regulators, and the promoter to be turned on to high levels (positive synergy). The removal of SUMO-conjugates then becomes a central step in the activation of complex promoters. We suggest that this can be achieved by SUMO-specific proteases, i.e. SENPs. Indeed we have shown that activation is possible when ectopically expressing SENP1 (PAPER III). It is noteworthy that in mammalian cells there are as much as seven different SUMO-proteases plus additional splice variants [289], as compared to only one or a few enzymes in each step of the conjugation reaction (see chapter 1.3.2.2). A directed and controlled recruitment of SUMO-proteases may therefore confer specificity to the activation switch process.

Having found that the synergy control is operating on c-Myb in a SUMO-dependent manner, we continued to dissect the mechanism. As mentioned HDACs did not seem to be involved in conveying synergy control in our system, since TSA treatment did not alter the synergy factor of wild-type c-Myb. Furthermore, conjugation of SUMO to c-Myb did not seem to have any influence on DNA-binding properties as evaluated by EMSA (PAPER III). We therefore chose to pursue the idea of a more intrinsic mechanism being involved in the switch from negative to positive synergy.

Since the model proposed (Fig 20) imply that the SUMO-conjugated array may exclude or suppress a transactivating function, we asked whether SUMO conjugated to c-Myb might have a more specific role in controlling the TAD functions of the factor. We started with addressing whether the region becoming sumoylated in c-Myb by itself might contribute to the transactivation properties of c-Myb in a SUMO-dependent manner. There have been a couple of reports in the past showing that this region may display transactivating activity in yeast [414, 415]. When we tested c-Myb NRD in Gal4 tethering assays, we observed a weak repressive effect as expected. However, when abrogating sumoylation by mutation or when co-transfecting with SENP1, NRD displayed a strong activating function (PAPER III). Furthermore, we showed that SUMO-negative full-length c-Myb was indeed more active than c-Myb with the NRD deleted, indicating that c-Myb 2KR is operating with two TADs (Fig 21). Finally, the SUMO-switch TAD in NRD could in fact rescue c-Myb transactivation when the central TAD was deleted (PAPER III). We therefore conclude that one aspect of the increased synergy observed for desumoylated c-Myb appears to be the derepression of the silenced transactivation domain in NRD (Fig 21). The NRD-TAD switch probably makes an important contribution to the large differences observed between wild-type and SUMO-negative c-Myb on complex promoters.

The presence of more than one activation function within a transcription factor is well known from the literature. The best studied examples might be the nuclear receptors which harbours two such functions AF1 and AF2, where AF1 activates in a ligand-independent fashion, while AF2 is ligand-dependent. Interestingly both AR and GR are SUMO-modified on lysines residing in AF1 [416]. As already mentioned, for GR this has been shown to impose synergy control on compound promoters [347]. The sumoylation sites in SF-1 also partly localise to a putative AF1 domain [417], while C/EBPα is sumoylated in the so-called "attenuator domain", sandwiched by two distinct transactivation domains [350, 418]. Hence, SUMO-switchable TAD/AFs might be a common theme in transcription factors subjected to synergy control. With two functional TADs operating in the same protein, the possibility exists that these two domains can synergize with each other, also in c-Myb. I fact we have data pointing in that direction. c-Myb ΔNRD which cannot be sumoylated, but at the same time lacks the NRD-TAD displays a SF that is significantly lower than full-length c-Myb 2KR (PAPER III). However, to really show that the two TADs in c-Myb synergize the c-Myb-ΔT1-2KR, with the central TAD deleted, should also be monitored on our synergy reporters.

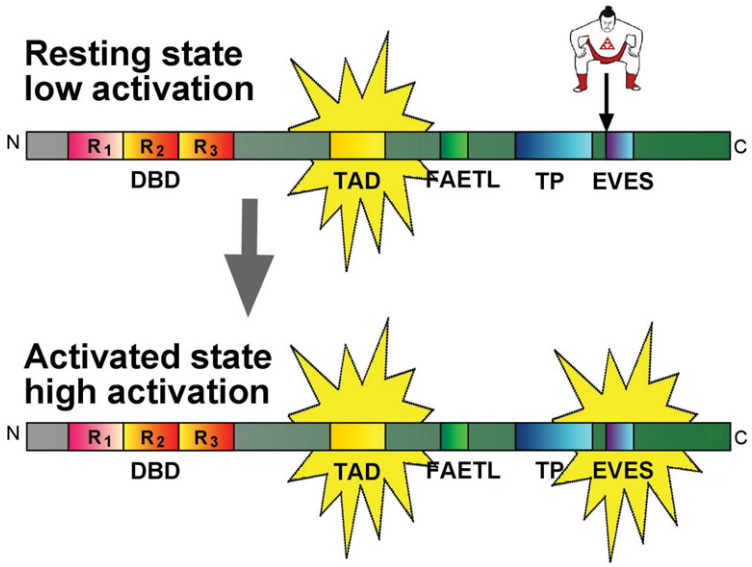

Figure 21 c-Myb has a second TAD in the NRD controlled by SUMO-conjugation. When c-Myb is sumoylated the transcription factor is operating with one TAD only. Upon desumoylation the second TAD becomes activated and increase both the activity and the synergistic potential of c-Myb

4.2.2 c-Myb binds SUMO: TAD contracts a repressive neighbour

In the last paper in this thesis we show that c-Myb harbours a motif that binds SUMO non-covalently, preferably SUMO-2 (PAPER IV). This motif (V_{267}NIV) is located at the very N-terminal border of the central transactivation domain. When destroying the SUMO-binding motif (SBM) by alanine substitutions (V_{267}NIV→A_{267}NAA), SUMO-binding is abrogated and the transactivational activity of c-Myb increases (PAPER IV). Thus, c-Myb seems to be repressed by SUMO-interaction at this site, and becomes derepressed when SUMO-binding is lost.

The initial mapping of the c-Myb transactivation domain was done employing Gal4 tethering assay with various Myb deletion constructs. This mapping defined the minimal TAD to be positioned between amino acid 275 and 325 in mouse and chicken c-Myb ([42, 55, 58]; same positions for human c-Myb). With the characterisation of the SBM (PAPER IV), we have identified a repressive determinant positioned right outside the minimal transactivation domain of c-Myb (amino acid residues 267-272). The c-Myb TAD is by far the domain in c-Myb that activates transcription most efficiently when fused to a heterologous DBD [58]. Still, we show that this ability can be even more enhanced by abrogating SUMO-binding (PAPER IV). The fact that a repressive motif like the c-Myb SBM is located head-to-tail with the transactivation domain is not unique. In fact several such examples may be found in the literature: In c-Myc an important repression element lies between the Myc-homology Box 1 (MB1) and Box 2 (MB2) TADs. In fact a second repression domain overlaps MB2 TAD itself (reviewed in [419]). Furthermore, the main transactivation domain AF1 in the androgen receptor harbours two sumoylation sites, which have been shown to repress ligand-dependent transactivation (reviewed in [420]). Likewise, having the possibility to tune the activity of the TAD through SUMO-binding undoubtedly adds dynamic to c-Myb-dependent gene activation.

In the work on synergy control we identified a second TAD in c-Myb (PAPER III; see chapter 4.2.1). This is another example of an activation function in c-Myb suppressed by SUMO, but in this instance SUMO-conjugation. In both cases obstruction of co-factor interaction could be part of the functional explanation. However, in the case of the SBM the repressive effect seems to be uncoupled from steric hindrance of the histone acetyl transferase p300, binding to c-Myb between amino acid residues 295-309 [66, 98, 99]. p300 co-activated TAD wild-type just as well as TAD ANAA when evaluated in Gal4 tethering assays (PAPER IV). Still, further experiments are needed to unravel the potential interplay between SBM-binding co-factors and CBP/p300.

Having identified a functional SBM in c-Myb, which when mutated caused derepression, we expected SBM to operate through the binding of a negatively acting sumoylated protein; either a SUMO-modified co-repressor binding in *trans*, or sumoylated NRD, binding in *cis*. Before we concentrated on these two non-mutually exclusive mechanisms, we had to rule out the possibility that the SBM somehow was involved the c-Myb SUMO-conjugation process. If so, abrogation of SUMO-binding might lower the sumoylation efficiency of c-Myb, and moreover, increase the transactivational activity. Western blot analyses of the different SBM mutants verified that the SUMO-binding negative ANAA mutant still was sumoylated (PAPER IV). In a more functional context we asked whether loss of SUMO-binding would affect the synergistic behaviour of c-Myb. By using the synergy reporters from PAPER III we could now get information relevant to identifying what mechanism is operating through the c-Myb SBM. Interestingly, calculation of the synergy factor showed that even though the A_{267}NAA mutant had the same activity as 2KR, its SF was well below 1.0, while the 2KR mutant had a SF ~ 4 (PAPER IV).

As shown in PAPER III, all mutations that eliminate or lower the SUMO-conjugation of c-Myb allow the protein to escape from the SC and to activate strongly on composite promoters. However, changes in SC as a result of abrogated SUMO-binding had so far not been addressed. Interestingly, the non-covalent binding surface of SUMO was recently demonstrated to be essential for SUMO-governed transcriptional (and synergistic) control [421]. Mutating residues in the second β-sheet and the following α-helix of SUMO, three of which (K33, K35 and R50) have been implicated in SUMO-binding [273, 276, 422], dramatically affected the ability of both SUMO-1 and -2 to inhibit transcription [421]. These findings support the idea that synergy control-mediating factors may be recruited to sumoylated transcription factors through non-covalent SUMO-binding [347]. However, since the c-Myb ANAA mutant revealed a synergy factor four-fold lower than the sumoylation negative mutant, we have no reasons to believe that synergy control can be imposed by sumoylated co-regulators when binding to SBMs in transcription factors on composite promoters. At least this seems not to be the case for c-Myb. Furthermore, since the SBM mutant ANAA was able to activate transcription to the same extent as c-Myb 2KR (Fig 22), we think that SUMO-binding and SUMO-conjugation/synergy control are two independent mechanisms operating on c-Myb. Moreover, it highlights the fact that even though synergy obviously promotes activity, these are two different phenomenons (Fig 22). Based on the data presented in Figure 22 and the previous reasoning, regulation of c-Myb activity through SUMO-binding might be more efficient on promoters with a moderate

number of REs, while the potential of regulation through SUMO-conjugation and synergy control increases with increasing number of REs.

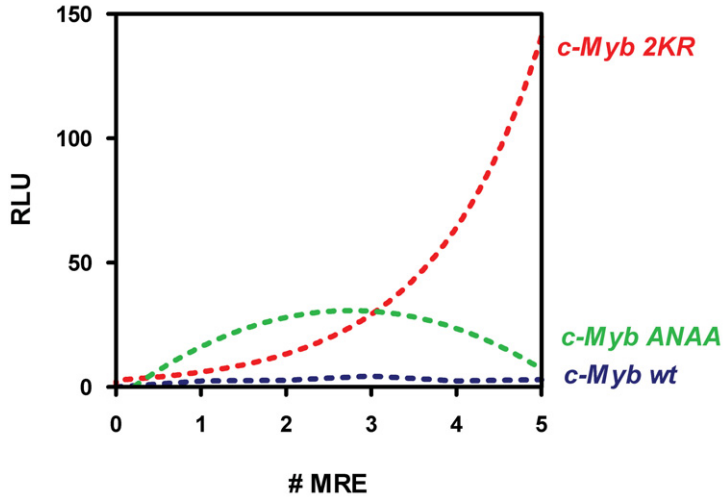

Figure 22 Activity and synergy - two different phenomenons. CV-1 cells were transfected with reporter plasmids containing increasing numbers of MREs (1-5), as indicated, and plasmids encoding c-Myb wild-type, SUMO-negative c-Myb 2KR, or SUMO-binding-negative c-Myb ANAA. The results are presented as relative luciferase units (RLU). The response curve for c-Myb ANAA was modelled based on activity measurements from 1×MRE, 3×MRE and 4×MRE reporters.

Even though the SBM did not seem to promote SC, we were intrigued by the fact that the sumoylation negative mutant 2KR and the SUMO-binding negative mutant ANAA seemed to have the same transactivational phenotype on several promoters (PAPER IV). We therefore wondered if the two SUMO-contacts might be involved in bridging parts of c-Myb. Such an explanation would substantiate the hypothesis of a fold-back mechanism between the EVES domain and the transactivation domain in c-Myb, suggested to conceal co-activator binding epitopes, thus lowering c-Myb activity [77, 423]. In one paper the transactivation domain of c-Myb was demonstrated to be activated in *trans* by c-Myb NRD when co-transfected in Gal4 tethering assays. This was in fact proposed to be caused by a universal cellular inhibitor, able bind both domains, being titrated out by the NRD [423]. Could this factor be SUMO? Peculiarly, B-Myb was not stimulated by c-Myb NRD [423]. However, from a SUMO perspective this would fit the fact that B-Myb harbours only one potential SUMO-binding motif which is identical to the c-Myb SBM1, shown to be non-functional (PAPER IV). Despite the indications of SUMO being involved, our data did not correspond with the activity pattern anticipated if the derepression was caused by disruption of an intramolecular association between the EVES domain and the SBM, bridged by SUMO. When introducing both the 2KR and ANAA mutations in the same construct, the transcriptional activity more than doubled, resulting in an extremely active phenotype (PAPER IV). Moreover, the ANAA mutant still had an activating effect when the NRD, including the SUMO-modified area of c-Myb, was deleted, and when studied in a TAD-only context (PAPER IV). Nevertheless, these experiments do not formally exclude the

possibility of a fold-back mechanism in c-Myb not involving SUMO or not leading to activity changes. Given that a binding between sumoylated NRD and SBM in TAD is neutral regarding c-Myb transactivational activity, the *trans* activation of c-Myb TAD by NRD observed by Vorbrueggen *et al*. [423] might just as well be caused by displacement of a co-repressor binding intermolecularly to the c-Myb SBM, resembling the data we obtained using conjugation-deficient SUMO (PAPER IV).

Mohan *et al*. recently demonstrated that SUMO conjugated to TDG (Thymine DNA Glycosylase) makes contact with an inherent SBM. This leads to conformational changes that abrogates DNA-binding [324, 326, 422]. In our study on synergy control in c-Myb we showed that constitutive SUMO-conjugation, mimicked by a c-Myb-SUMO fusion, did not alter the DNA-binding properties of the protein (PAPER III). This indicates that the c-Myb SUMO moieties do not act in *cis*, when repressing c-Myb activity. Conversely, when DNA was added to the interaction buffers in the SUMO-binding study, we were still able to pull down c-Myb using GST-SUMO-2 (PAPER IV). Hence, DNA-binding does not seem to alter the SUMO binding properties of c-Myb. Taken together this further substantiates our conclusion that if an intramolecular SUMO-bridge does exist in c-Myb, it will most likely not have any impact on neither transactivation, nor DNA-binding.

4.2.3 Keeping c-Myb busy: The PML-NB as a c-Myb co-factor distributor.

Having established that c-Myb binds SUMO, most likely in the form of another sumoylated negatively acting protein, we chose to focus on the tumour suppressor Promyelocytic Leukemia (PML) protein. Several functions have been suggested for PML and the PML nuclear bodies (PML-NBs) [424]. However, the prevailing model is that PML functions as a kind of storage, sequestering nuclear proteins. Recently, both sumoylation and SUMO-binding have been shown to regulate nucleation of the PML protein into nuclear bodies [352, 354]. Furthermore, the presence of unoccupied SUMO-moieties and SBMs on the surface of the PML NBs allows for sumoylated and/or SBM-containing factors to be recruited to these complexes [354]. Several papers have shown that protein-protein interactions associated with the PML-NBs, as well as the bodies themselves, are highly dynamic [328, 425]. Thus, combined with the fact that these NBs also are sites for post-translational modifications (reviewed in [424]), the PML-NBs should be recognized as highly active protein distribution centres.

We have previously shown that PML interacts with c-Myb and recruits it to PML nuclear bodies [117]. When comparing the co-localization of c-Myb wild-type and SUMO-

Interacting protein	SUMO-modified	SUMO-binding
Daxx	Yes	Yes
HDAC1	Yes	Yes
HDAC2	(Yes)	Yes
HDAC3	No	(Yes)
HIPK2	Yes	Yes
N-CoR 1	Yes	(Yes)
N-CoR 2	(Yes)	-
SP100	Yes	Yes
PML	Yes	Yes
TDG	Yes	Yes
TOPORS	Yes	Yes
ARNT	Yes	-
CBP/p300	Yes	-
c-Fos	Yes	-
c-Jun	Yes	-
GATA2	Yes	-
Mdm2	Yes	-
p53	Yes	-
p73	Yes	-
PLZF	Yes	-
pRb	Yes	-
RFP	Yes	-
Sin3A	Yes	-
Smad 2	Yes	-
Smad 3	Yes	-
Sp 1	Yes	-
SRF	Yes	-
TIF1-α	Yes	-
SENP2	-	Yes
c-Ski	-	-
FLASH	-	-
p38 MAPK	-	-

Table 3 PML-NB interacting proteins. A selection of mammalian proteins reported to be recruited to PML nuclear bodies (www.gene-profiles.org). Evidence of SUMO modification and SUMO-binding properties are indicated. (Yes): indirect evidence, e.g. by sequence homology; –: not determined.

contact mutants (ANAA, 2KR and ANAA 2KR) with PML, we could see that c-Myb is recruited to PML-NBs in a SUMO contact-independent manner (PAPER IV). Dahle *et al*. showed that the interaction between c-Myb and PML appeared to be independent of c-Myb sumoylation, although a quantitative difference was not excluded [117]. Our new data supports this, and extend the notion of a SUMO-independent recruitment of c-Myb to PML-NBs to include also SUMO-binding (PAPER IV). Curiously, this did not correlate with functional data showing that c-Myb wild-type is activated by ectopic PML expression, while the SUMO-binding and sumoylation-negative mutants have lost most of this potential as the

relative PML co-activation decreased with the elimination of functional SUMO-contacts (PAPER IV).

We believe that the co-activation of wild-type c-Myb might be caused by PML-NBs sequestering negatively acting co-factors operating through the SBM or the conjugated SUMO moieties. Consequently, the reduced PML co-activation seen with the SUMO-contact mutants might be due to co-repressor interactions already being broken. A similar mechanism has been proposed for the glucocorticoid receptor, for which PML functions as a co-activator through sequestering the SUMO-binding co-repressor Daxx [346, 426]. Whether Daxx may function as a co-repressor also for c-Myb is not yet known. However thinking along these lines, we should be looking for PML/PML-NB-interacting proteins, known to repress transcription, that either are sumoylated or able to bind SUMO. Such factors would be candidates for SUMO-binding or SUMO modification-dependent repressors of c-Myb transactivational activity. Daxx is only one out of more than 50 proteins reported to interact with PML[3]. In table 3, I have listed some of these proteins and included information on SUMO-modification and SUMO-binding properties. Notably, not all the proteins in this table are known to function as co-repressors. However, Daxx, HDAC1-3, HIPK2, N-CoR, as well as SP100 and PML it self have been reported to repress transcription [346, 424, 427-429]. Interestingly, HIPK2, N-CoR, Sin3A and c-Ski have been reported to interact with c-Myb and negatively regulate Myb-dependent transactivation [90, 109]. However, none of them have been reported to interact directly with the EVES domain or the SBM. In their work on SUMO-2/3 conjugation of c-Myb Sramko *et al*. observed a derepression of c-Myb activity using trichostatin A [83], indicating that we might be looking at an HDAC being recruited to the SUMO conjugates. Still, these are only speculations, and future work will hopefully identify SUMO-contact dependent co-repressors acting on c-Myb.

4.3 SUMMARY AND FUTURE PERSPECTIVES

In the four papers included in this thesis we have described two new regulatory mechanisms and two novel co-activators of c-Myb. The proto-oncoprotein c-Myb is known to be an activator of transcription. Hence, the identification of two novel co-activators of c-Myb fits well with the current understanding of this protein. Still, repressive mechanisms might be just as central in c-Myb biology as an extensive repertoire of positively acting co-factors. In

[3] www.gene-profiles.org

the end opposing mechanisms are probably integrated to ensure an accurate regulation of c-Myb activity.

4.3.1 FLASH and Mi-2α meet SUMO

With the knowledge we have generated on both FLASH and Mi-2α action as well as SUMO-binding, SUMO-conjugation and synergy control, it should be possible to search for both interdependency and mutually exclusiveness between these mechanisms of action. Our study on synergy control indicated that the multiplicity of TADs on a given promoter might be an important aspect in promoting positive synergy (PAPER III). A natural question would then be if co-activators interacting with c-Myb on non-TAD surfaces, like FLASH and Mi-2α, will provide c-Myb with a second (or even third) TAD, and thereby result in positive synergy. Furthermore, could such interactions compensate for the central TAD in c-Myb if deleted? In PAPER I we presented data showing that desumoylation of c-Myb potentiated the Mi-2α-c-Myb co-activation, alternatively, that SUMO-modification reduced this cooperation. It would be relevant to see if the same held true for FLASH. Moreover, p300 could be included in such an experiment. Another question worth asking is whether some of these interactions and modifications are mutually exclusive. Even though we do not have any observations supporting SUMO-governed intramolecular interactions in c-Myb, it would be interesting to examine whether sumoylated c-Myb is able to interact with FLASH and Mi-2α. After all, FLASH seemed to interact more strongly with the C-terminally deleted c-Myb[1-443] than the wild-type (PAPER II). Furthermore, will FLASH and/or Mi-2α inhibit sumoylation of c-Myb? Notably, we have not seen such effects for the well established c-Myb co-activator p300, as evaluated by western blotting (data not shown).

Some of the same questions might be relevant for SUMO-binding. Will the SBM-mutant A_{267}NAA have an increased affinity for Mi-2α, hence lead to a better co-activation? What about the I_{267}NII mutant? In PAPER II we showed that FLASH seemed to bind just as efficient to v-Myb (INII) as c-Myb[1-443] (VNIV), however these observations might not be relevant due to the mutations and deletion in v-Myb DBD and the lack of comparable amounts of SUMO or sumoylated proteins in the interaction reaction. Hence, further studies are needed. Conversely, is c-Myb able to interact with SUMO when Mi-2α or FLASH is bound? To answer this question employing Mi-2α or FLASH-Myb fusion proteins might be a strategy. Finally, several of these questions could be addressed in a more physiological setting, using chromatin immunoprecipitation (ChIP) and re-ChIP to look for co-occupancy

on relevant target genes. Alternatively, the precipitated DNA from such assays could be cloned and sequenced to identify candidate promoters.

4.3.2 SUMO-binding, SUMO-conjugation and the transforming properties of c-Myb.

As discussed in chapter 4.1.5 it is important to keep in mind that some of the mechanisms unravelled in these studies may unleash the transforming potential of c-Myb when damaged or deregulated. The truncated and mutated AMV v-Myb causes rapid and fatal monoblastic leukemia in chicken (reviewed in [16]). Moreover, deregulation and duplication of *MYB* are probably involved in the development human T-ALL [242-244]. A common theme in both chicken and human leukemias involving c-*myb*/*MYB* anomalies is that the overall Myb-activity seems to be increased. In PAPER III and IV we describe two SUMO-related mechanisms involved in dampening c-Myb activity. Interestingly, both the SUMO-binding motif and the SUMO-modified area of c-Myb are hit by mutations and deletions in the transforming v-Myb protein. Notably, v-Myb was shown to have the highest synergy factor (~5) among the Myb-proteins tested in PAPER III. Moreover, the combination of mutations abrogating both SUMO-conjugation and binding in a c-Myb background resulted in an extremely active phenotype (PAPER IV).

It has been demonstrated that c-Myb with C-terminal truncations can transform avian yolk sac and bone marrow [69] and murine foetal liver cultures [430, 431]. This opens up the possibility that lost SUMO-modification and/or synergy control might be involved in the oncogenic activation c-Myb. Concerning SUMO-binding, the v-Myb protein seems to have retained this property (PAPER IV). Moreover, a linker insertion mutagenesis destroying the SBM in v-Myb resulted in a mutant (I_{202}GPNII) with somewhat reduced capacity to transform avian yolk sack [432]. In a recent genome-wide ENU mutagenesis screening study, two point mutations in c-*myb* were identified that were able to rescue mice from a myeloproliferative syndrome [166]. Interestingly, these point mutations generated two potential SUMO-binding motifs in c-Myb and resulted in proteins with lower transactivation potential. This might indicate that changing the SUMO-binding properties of c-Myb may have biological effects.

In recent years it has become apparent that deregulation of the sumoylation system as well as mutations or deletions of SUMO-acceptor sites are associated with the development of human diseases, including many types of cancers [251]. Therefore, we would like to pursue the possible link between lost SUMO regulation of c-Myb and transformation by this proto-oncoprotein. We have recently initiated a joint effort with the

research group of Prof. T.J. Gonda at the University of Queensland, where we are going to address the transforming properties of our different SUMO-contact mutants. Hopefully, this will result in new knowledge about the potential role of c-Myb and SUMO in disease.

5. REFERENCES

1. Lander, E.S. et al. (2001) Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921.
2. Venter, J.C. et al. (2001) The sequence of the human genome. *Science* **291**, 1304-51.
3. Brentani, H. et al. (2003) The generation and utilization of a cancer-oriented representation of the human transcriptome by using expressed sequence tags. *Proc Natl Acad Sci U S A* **100**, 13418-23.
4. Emery, J.G., Ohlstein, E.H. & Jaye, M. (2001) Therapeutic modulation of transcription factor activity. *Trends Pharmacol Sci* **22**, 233-40.
5. Tucker, C.L., Gera, J.F. & Uetz, P. (2001) Towards an understanding of complex protein networks. *Trends Cell Biol* **11**, 102-6.
6. Brivanlou, A.H. & Darnell, J.E.J. (2002) Signal transduction and the control of gene expression. *Science* **295**, 813-8.
7. Stracke, R., Werber, M. & Weisshaar, B. (2001) The R2R3-MYB gene family in Arabidopsis thaliana. *Curr Opin Plant Biol* **4**, 447-56.
8. Ganter, B. & Lipsick, J.S. (1999) Myb and oncogenesis. *Adv Cancer Res* **76**, 21-60.
9. Jin, H. & Martin, C. (1999) Multifunctionality and diversity within the plant MYB-gene family. *Plant Mol Biol* **41**, 577-85.
10. Lipsick, J.S. (1996) One billion years of Myb. *Oncogene* **13**, 223-235.
11. Lemon, B. & Tjian, R. (2000) Orchestrated response: a symphony of transcription factors for gene control. *Genes Dev* **14**, 2551-69.
12. Khorasanizadeh, S. (2004) The nucleosome: from genomic organization to genomic regulation. *Cell* **116**, 259-72.
13. Eberharter, A. & Becker, P.B. (2004) ATP-dependent nucleosome remodelling: factors and functions. *J Cell Sci* **117**, 3707-11.
14. Margueron, R., Trojer, P. & Reinberg, D. (2005) The key to development: interpreting the histone code? *Curr Opin Genet Dev* **15**, 163-76.
15. Werner, T. (2001) The promoter connection. *Nat Genet* **29**, 105-6.
16. Lipsick, J.S. & Wang, D.M. (1999) Transformation by v-Myb. *Oncogene* **18**, 3047-55.
17. Duesberg, P.H., Bister, K. & Moscovici, C. (1980) Genetic structure of avian myeloblastosis virus, released from transformed myeloblasts as a defective virus particle. *Proc Natl Acad Sci U S A* **77**, 5120-4.
18. Souza, L.M., Strommer, J.N., Hillyard, R.L., Komaromy, M.C. & Baluda, M.A. (1980) Cellular sequences are present in the presumptive avian myeloblastosis virus genome. *Proc Natl Acad Sci U S A* **77**, 5177-81.
19. Klempnauer, K.H., Gonda, T.J. & Bishop, J.M. (1982) Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: the architecture of a transduced oncogene. *Cell* **31**, 453-63.
20. Lu, C.A., Ho, T.H., Ho, S.L. & Yu, S.M. (2002) Three Novel MYB Proteins with One DNA Binding Repeat Mediate Sugar and Hormone Regulation of alpha-Amylase Gene Expression. *Plant Cell* **14**, 1963-80.
21. Nomura, N., Takahashi, M., Matsui, M., Ishii, S., Date, T., Sasamoto, S. & Ishizaki, R. (1988) Isolation of human cDNA clones of myb-related genes, A-myb and B-myb. *Nucleic Acids Res* **16**, 11075-89.
22. Foos, G., Grimm, S. & Klempnauer, K.H. (1994) The chicken A-myb protein is a transcriptional activator. *Oncogene* **9**, 2481-8.
23. Sala, A. (2005) B-MYB, a transcription factor implicated in regulating cell cycle, apoptosis and cancer. *Eur J Cancer* **41**, 2479-84.
24. Weston, K. (1998) Myb proteins in life, death and differentiation. *Curr Opin Genet Dev* **8**, 76-81.
25. Saville, M.K. & Watson, R.J. (1998) B-Myb: a key regulator of the cell cycle. *Adv Cancer Res* **72**, 109-40.
26. Moscovici, M.G., Jurdic, P., Samarut, J., Gazzolo, L., Mura, C.V. & Moscovici, C. (1983) Characterization of the hemopoietic target cells for the avian leukemia virus E26. *Virology* **129**, 65-78.
27. Graf, T., McNagny, K., Brady, G. & Frampton, J. (1992) Chicken 'erythroid' cells transformed by the Gag-Myb-Ets-encoding E26 leukemia virus are multipotent. *Cell* **70**, 201-13.
28. Anton, I.A. & Frampton, J. (1988) Tryptophans in myb proteins. *Nature* **336**, 719.
29. Kanei-Ishii, C., Sarai, A., Sawazaki, T., Nakagoshi, H., He, D.N., Ogata, K., Nishimura, Y. & Ishii, S. (1990) The tryptophan cluster: a hypothetical structure of the DNA-binding domain of the myb protooncogene product. *J Biol Chem* **265**, 19990-5.
30. Gabrielsen, O.S., Sentenac, A. & Fromageot, P. (1991) Specific DNA binding by c-Myb: evidence for a double helix-turn-helix- related motif. *Science* **253**, 1140-3.

31. Frampton, J., Leutz, A., Gibson, T. & Graf, T. (1989) DNA-binding domain ancestry. *Nature* **342**, 134.
32. Frampton, J., Gibson, T.J., Ness, S.A., Doderlein, G. & Graf, T. (1991) Proposed structure for the DNA-binding domain of the Myb oncoprotein based on model building and mutational analysis. *Protein Eng* **4**, 891-901.
33. Jamin, N., Gabrielsen, O.S., Gilles, N., Lirsac, P.N. & Toma, F. (1993) Secondary structure of the DNA-binding domain of the c-Myb oncoprotein in solution. A multidimensional double and triple heteronuclear NMR study. *Eur J Biochem* **216**, 147-54.
34. Ogata, K., Hojo, H., Aimoto, S., Nakai, T., Nakamura, H., Sarai, A., Ishii, S. & Nishimura, Y. (1992) Solution structure of a DNA-binding unit of Myb: a helix-turn-helix- related motif with conserved tryptophans forming a hydrophobic core. *Proc Natl Acad Sci U S A* **89**, 6428-32.
35. Ogata, K., Morikawa, S., Nakamura, H., Sekikawa, A., Inoue, T., Kanai, H., Sarai, A., Ishii, S. & Nishimura, Y. (1994) Solution structure of a specific DNA complex of the Myb DNA-binding domain with cooperative recognition helices. *Cell* **79**, 639-48.
36. Ogata, K., Morikawa, S., Nakamura, H., Hojo, H., Yoshimura, S., Zhang, R., Aimoto, S., Ametani, Y., Hirata, Z. & Sarai, A. (1995) Comparison of the free and DNA-complexed forms of the DNA-binding domain from c-Myb. *Nat Struct Biol* **2**, 309-20.
37. Carr, M.D., Wollborn, U., McIntosh, P.B., Frenkiel, T.A., McCormick, J.E., Bauer, C.J., Klempnauer, K.H. & Feeney, J. (1996) Structure of the B-Myb DNA-binding domain in solution and evidence for multiple conformations in the region of repeat-2 involved in DNA binding: implications for sequence-specific DNA binding by Myb proteins. *Eur J Biochem* **235**, 721-35.
38. Myrset, A.H., Bostad, A., Jamin, N., Lirsac, P.N., Toma, F. & Gabrielsen, O.S. (1993) DNA and redox state induced conformational changes in the DNA-binding domain of the Myb oncoprotein. *EMBO J* **12**, 4625-33.
39. Tahirov, T.H. et al. (2002) Mechanism of c-Myb-C/EBP beta cooperation from separated sites on a promoter. *Cell* **108**, 57-70.
40. Howe, K.M., Reakes, C.F. & Watson, R.J. (1990) Characterization of the sequence-specific interaction of mouse c-myb protein with DNA. *EMBO J* **9**, 161-9.
41. Garcia, A., LaMontagne, K., Reavis, D., Stober-Grasser, U. & Lipsick, J.S. (1991) Determinants of sequence-specific DNA-binding by p48v-myb. *Oncogene* **6**, 265-73.
42. Sakura, H., Kanei-Ishii, C., Nagase, T., Nakagoshi, H., Gonda, T.J. & Ishii, S. (1989) Delineation of three functional domains of the transcriptional activator encoded by the c-myb protooncogene. *Proc Natl Acad Sci U S A* **86**, 5758-62.
43. Hegvold, A.B. & Gabrielsen, O.S. (1996) The importance of the linker connecting the repeats of the c-Myb oncoprotein may be due to a positioning function. *Nucleic Acids Res* **24**, 3990-5.
44. Biedenkapp, H., Borgmeyer, U., Sippel, A.E. & Klempnauer, K.H. (1988) Viral myb oncogene encodes a sequence-specific DNA-binding activity. *Nature* **335**, 835-7.
45. Howe, K.M. & Watson, R.J. (1991) Nucleotide preferences in sequence-specific recognition of DNA by c-myb protein. *Nucleic Acids Res* **19**, 3913-9.
46. Weston, K. (1992) Extension of the DNA binding consensus of the chicken c-Myb and v-Myb proteins. *Nucleic Acids Res* **20**, 3043-9.
47. Ganter, B., Chao, S.T. & Lipsick, J.S. (1999) Transcriptional activation by the myb proteins requires a specific local promoter structure. *FEBS Lett* **460**, 401-10.
48. Berge, T., Bergholtz, S.L., Andersson, K.B. & Gabrielsen, O.S. (2001) A novel yeast system for in vivo selection of recognition sequences: defining an optimal c-Myb-responsive element. *Nucleic Acids Res* **29**, E99.
49. Aasland, R., Stewart, A.F. & Gibson, T. (1996) The SANT domain: a putative DNA-binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressor N-CoR and TFIIIB. *Trends Biochem Sci* **21**, 87-8.
50. Boyer, L.A., Langer, M.R., Crowley, K.A., Tan, S., Denu, J.M. & Peterson, C.L. (2002) Essential role for the SANT domain in the functioning of multiple chromatin remodeling enzymes. *Mol Cell* **10**, 935-42.
51. Yu, J., Li, Y., Ishizuka, T., Guenther, M.G. & Lazar, M.A. (2003) A SANT motif in the SMRT corepressor interprets the histone code and promotes histone deacetylation. *Embo J* **22**, 3403-10.
52. Sterner, D.E., Wang, X., Bloom, M.H., Simon, G.M. & Berger, S.L. (2002) The SANT domain of Ada2 is required for normal acetylation of histones by the yeast SAGA complex. *J Biol Chem* **277**, 8178-86.
53. Boyer, L.A., Latek, R.R. & Peterson, C.L. (2004) The SANT domain: a unique histone-tail-binding module? *Nat Rev Mol Cell Biol* **5**, 158-63.
54. Mo, X., Kowenz-Leutz, E., Laumonnier, Y., Xu, H. & Leutz, A. (2005) Histone H3 tail positioning and acetylation by the c-Myb but not the v-Myb DNA-binding SANT domain. *Genes Dev* **19**, 2447-57.

55. Weston, K. & Bishop, J.M. (1989) Transcriptional activation by the v-myb oncogene and its cellular progenitor, c-myb. *Cell* **58**, 85-93.
56. Ibanez, C.E. & Lipsick, J.S. (1990) Trans activation of gene expression by v-myb. *Mol Cell Biol* **10**, 2285-93.
57. Klempnauer, K.H., Arnold, H. & Biedenkapp, H. (1989) Activation of transcription by v-myb: evidence for two different mechanisms. *Genes Dev* **3**, 1582-9.
58. Kalkbrenner, F., Guehmann, S. & Moelling, K. (1990) Transcriptional activation by human c-myb and v-myb genes. *Oncogene* **5**, 657-61.
59. Chen, R.H., Fields, S. & Lipsick, J.S. (1995) Dissociation of transcriptional activation and oncogenic transformation by v-Myb. *Oncogene* **11**, 1771-9.
60. Fu, S.L. & Lipsick, J.S. (1996) FAETL motif required for leukemic transformation by v-Myb. *J Virol* **70**, 5600-10.
61. Heery, D.M., Kalkhoven, E., Hoare, S. & Parker, M.G. (1997) A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* **387**, 733-6.
62. Torchia, J., Rose, D.W., Inostroza, J., Kamei, Y., Westin, S., Glass, C.K. & Rosenfeld, M.G. (1997) The transcriptional co-activator p/CIP binds CBP and mediates nuclear- receptor function. *Nature* **387**, 677-84.
63. Darimont, B.D., Wagner, R.L., Apriletti, J.W., Stallcup, M.R., Kushner, P.J., Baxter, J.D., Fletterick, R.J. & Yamamoto, K.R. (1998) Structure and specificity of nuclear receptor-coactivator interactions. *Genes Dev* **12**, 3343-56.
64. Nolte, R.T., Wisely, G.B., Westin, S., Cobb, J.E., Lambert, M.H., Kurokawa, R., Rosenfeld, M.G., Willson, T.M., Glass, C.K. & Milburn, M.V. (1998) Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. *Nature* **395**, 137-43.
65. Demarest, S.J., Martinez-Yamout, M., Chung, J., Chen, H., Xu, W., Dyson, H.J., Evans, R.M. & Wright, P.E. (2002) Mutual synergistic folding in recruitment of CBP/p300 by p160 nuclear receptor coactivators. *Nature* **415**, 549-53.
66. Zor, T., De Guzman, R.N., Dyson, H.J. & Wright, P.E. (2004) Solution structure of the KIX domain of CBP bound to the transactivation domain of c-Myb. *J Mol Biol* **337**, 521-34.
67. Zor, T., Mayr, B.M., Dyson, H.J., Montminy, M.R. & Wright, P.E. (2002) Roles of phosphorylation and helix propensity in the binding of the KIX domain of CREB-binding protein by constitutive (c-Myb) and inducible (CREB) activators. *J Biol Chem* **277**, 42241-8.
68. Parker, D., Rivera, M., Zor, T., Henrion-Caude, A., Radhakrishnan, I., Kumar, A., Shapiro, L.H., Wright, P.E., Montminy, M. & Brindle, P.K. (1999) Role of secondary structure in discrimination between constitutive and inducible activators. *Mol Cell Biol* **19**, 5601-7.
69. Hu, Y.L., Ramsay, R.G., Kanei-Ishii, C., Ishii, S. & Gonda, T.J. (1991) Transformation by carboxyl-deleted Myb reflects increased transactivating capacity and disruption of a negative regulatory domain. *Oncogene* **6**, 1549-53.
70. Kanei-Ishii, C., MacMillan, E.M., Nomura, T., Sarai, A., Ramsay, R.G., Aimoto, S., Ishii, S. & Gonda, T.J. (1992) Transactivation and transformation by Myb are negatively regulated by a leucine-zipper structure. *Proc Natl Acad Sci U S A* **89**, 3088-92.
71. Favier, D. & Gonda, T.J. (1994) Detection of proteins that bind to the leucine zipper motif of c-Myb. *Oncogene* **9**, 305-11.
72. Tavner, F.J. et al. (1998) Molecular cloning reveals that the p160 Myb-binding protein is a novel, predominantly nucleolar protein which may play a role in transactivation by Myb. *Mol Cell Biol* **18**, 989-1002.
73. Ebneth, A., Adermann, K. & Wolfes, H. (1994) Does a synthetic peptide containing the leucine-zipper domain of c-myb form an alpha-helical structure in solution? *FEBS Lett* **337**, 265-8.
74. Nomura, T., Sakai, N., Sarai, A., Sudo, T., Kanei-Ishii, C., Ramsay, R.G., Favier, D., Gonda, T.J. & Ishii, S. (1993) Negative autoregulation of c-Myb activity by homodimer formation through the leucine zipper. *J Biol Chem* **268**, 21914-23.
75. Krieg, J., Oelgeschlager, M., Janknecht, R. & Luscher, B. (1995) High affinity DNA binding of native full length c-Myb and differential proteolytic sensitivity of its N- and C-terminal domains. *Oncogene* **10**, 2221-8.
76. Ness, S.A. (1996) The Myb oncoprotein: regulating a regulator. *Biochim Biophys Acta* **1288**, F123-139.
77. Dubendorff, J.W., Whittaker, L.J., Eltman, J.T. & Lipsick, J.S. (1992) Carboxy-terminal elements of c-Myb negatively regulate transcriptional activation in cis and in trans. *Genes Dev* **6**, 2524-35.
78. Sano, Y. & Ishii, S. (2001) Increased affinity of c-Myb for CREB-binding protein (CBP) after CBP-induced acetylation. *J Biol Chem* **276**, 3674-82.

79. Tomita, A., Towatari, M., Tsuzuki, S., Hayakawa, F., Kosugi, H., Tamai, K., Miyazaki, T., Kinoshita, T. & Saito, H. (2000) c-Myb acetylation at the carboxyl-terminal conserved domain by transcriptional co-activator p300. *Oncogene* **19**, 444-51.
80. Kanei-Ishii, C., Nomura, T., Tanikawa, J., Ichikawa-Iwata, E. & Ishii, S. (2004) Differential sensitivity of v-Myb and c-Myb to Wnt-1-induced protein degradation. *J Biol Chem* **279**, 44582-9.
81. Dahle, O., Andersen, T.O., Nordgard, O., Matre, V., Del Sal, G. & Gabrielsen, O.S. (2003) Transactivation properties of c-Myb are critically dependent on two SUMO-1 acceptor sites that are conjugated in a PIASy enhanced manner. *Eur J Biochem* **270**, 1338-48.
82. Bies, J., Markus, J. & Wolff, L. (2002) Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity. *J Biol Chem* **277**, 8999-9009.
83. Sramko, M., Markus, J., Kabat, J., Wolff, L. & Bies, J. (2006) Stress-induced inactivation of the c-Myb transcription factor through conjugation of SUMO-2/3 proteins. *J Biol Chem* **281**, 40065-75.
84. Aziz, N., Wu, J., Dubendorff, J.W., Lipsick, J.S., Sturgill, T.W. & Bender, T.P. (1993) c-Myb and v-Myb are differentially phosphorylated by p42mapk in vitro. *Oncogene* **8**, 2259-65.
85. Aziz, N., Miglarese, M.R., Hendrickson, R.C., Shabanowitz, J., Sturgill, T.W., Hunt, D.F. & Bender, T.P. (1995) Modulation of c-Myb-induced transcription activation by a phosphorylation site near the negative regulatory domain. *Proc Natl Acad Sci U S A* **92**, 6429-33.
86. Vorbrueggen, G., Lovric, J. & Moelling, K. (1996) Functional analysis of phosphorylation at serine 532 of human c-Myb by MAP kinase. *Biol Chem* **377**, 721-30.
87. Miglarese, M.R., Richardson, A.F., Aziz, N. & Bender, T.P. (1996) Differential regulation of c-Myb-induced transcription activation by a phosphorylation site in the negative regulatory domain. *J Biol Chem* **271**, 22697-705.
88. Bies, J., Feikova, S., Bottaro, D.P. & Wolff, L. (2000) Hyperphosphorylation and increased proteolytic breakdown of c-Myb induced by the inhibition of Ser/Thr protein phosphatases. *Oncogene* **19**, 2846-54.
89. Keough, R.A., Macmillan, E.M., Lutwyche, J.K., Gardner, J.M., Tavner, F.J., Jans, D.A., Henderson, B.R. & Gonda, T.J. (2003) Myb-binding protein 1a is a nucleocytoplasmic shuttling protein that utilizes CRM1-dependent and independent nuclear export pathways. *Exp Cell Res* **289**, 108-23.
90. Nomura, T., Tanikawa, J., Akimaru, H., Kanei-Ishii, C., Ichikawa-Iwata, E., Khan, M.M., Ito, H. & Ishii, S. (2004) Oncogenic activation of c-Myb correlates with a loss of negative regulation by TIF1beta and Ski. *J Biol Chem* **279**, 16715-26.
91. Ladendorff, N.E., Wu, S. & Lipsick, J.S. (2001) BS69, an adenovirus E1A-associated protein, inhibits the transcriptional activity of c-Myb. *Oncogene* **20**, 125-32.
92. Ramsay, R.G., Ishii, S. & Gonda, T.J. (1991) Increase in specific DNA binding by carboxyl truncation suggests a mechanism for activation of Myb. *Oncogene* **6**, 1875-9.
93. Dash, A.B., Orrico, F.C. & Ness, S.A. (1996) The EVES motif mediates both intermolecular and intramolecular regulation of c-Myb. *Genes Dev* **10**, 1858-69.
94. Kiewitz, A. & Wolfes, H. (1997) Mapping of protein-protein interactions between c-myb and its coactivator CBP by a new phage display technique. *FEBS Lett* **415**, 258-62.
95. Ness, S.A. (1999) Myb binding proteins: regulators and cohorts in transformation. *Oncogene* **18**, 3039-46.
96. Ness, S.A., Kowenz-Leutz, E., Casini, T., Graf, T. & Leutz, A. (1993) Myb and NF-M: combinatorial activators of myeloid genes in heterologous cell types. *Genes Dev* **7**, 749-59.
97. Burk, O., Mink, S., Ringwald, M. & Klempnauer, K.H. (1993) Synergistic activation of the chicken mim-1 gene by v-myb and C/EBP transcription factors. *EMBO J* **12**, 2027-38.
98. Dai, P., Akimaru, H., Tanaka, Y., Hou, D.X., Yasukawa, T., Kanei-Ishii, C., Takahashi, T. & Ishii, S. (1996) CBP as a transcriptional coactivator of c-Myb. *Genes Dev* **10**, 528-40.
99. Oelgeschlager, M., Janknecht, R., Krieg, J., Schreek, S. & Luscher, B. (1996) Interaction of the co-activator CBP with Myb proteins: effects on Myb-specific transactivation and on the cooperativity with NF-M. *Embo J* **15**, 2771-80.
100. Mink, S., Kerber, U. & Klempnauer, K.H. (1996) Interaction of C/EBPbeta and v-Myb is required for synergistic activation of the mim-1 gene. *Mol Cell Biol* **16**, 1316-25.
101. Ganter, B., Fu, S.l. & Lipsick, J.S. (1998) D-type cyclins repress transcriptional activation by the v-Myb but not the c-Myb DNA-binding domain. *EMBO J* **17**, 255-68.
102. Leverson, J.D. & Ness, S.A. (1998) Point mutations in v-Myb disrupt a cyclophilin-catalyzed negative regulatory mechanism. *Mol Cell* **1**, 203-11.
103. Ying, G.G., Proost, P., van Damme, J., Bruschi, M., Introna, M. & Golay, J. (2000) Nucleolin, a novel partner for the Myb transcription factor family that regulates their activity. *J Biol Chem* **275**, 4152-8.
104. Hedge, S.P., Kumar, A., Kurschner, C. & Shapiro, L.H. (1998) c-Maf interacts with c-Myb to regulate transcription of an early myeloid gene during differentiation. *Mol Cell Biol* **18**, 2729-37.

105. Peng, S., Lalani, S., Leavenworth, J.W., Ho, I.C. & Pauza, M.E. (2007) c-Maf interacts with c-Myb to down-regulate Bcl-2 expression and increase apoptosis in peripheral CD4 cells. *Eur J Immunol* **37**, 2868-80.
106. Tillmanns, S., Otto, C., Jaffray, E., Du Roure, C., Bakri, Y., Vanhille, L., Sarrazin, S., Hay, R.T. & Sieweke, M.H. (2007) SUMO modification regulates MafB-driven macrophage differentiation by enabling Myb-dependent transcriptional repression. *Mol Cell Biol* **27**, 5554-64.
107. Kanei-Ishii, C., Tanikawa, J., Nakai, A., Morimoto, R.I. & Ishii, S. (1997) Activation of heat shock transcription factor 3 by c-Myb in the absence of cellular stress. *Science* **277**, 246-8.
108. Winn, L.M., Lei, W. & Ness, S.A. (2003) Pim-1 phosphorylates the DNA binding domain of c-Myb. *Cell Cycle* **2**, 258-62.
109. Kanei-Ishii, C. et al. (2004) Wnt-1 signal induces phosphorylation and degradation of c-Myb protein via TAK1, HIPK2, and NLK. *Genes Dev* **18**, 816-29.
110. Morita, Y., Kanei-Ishii, C., Nomura, T. & Ishii, S. (2005) TRAF7 Sequesters c-Myb to the Cytoplasm by Stimulating Its Sumoylation. *Mol Biol Cell* **16**, 5433-44.
111. Pfitzner, E., Kirfel, J., Becker, P., Rolke, A. & Schule, R. (1998) Physical interaction between retinoic acid receptor and the oncoprotein myb inhibits retinoic acid-dependent transactivation. *Proc Natl Acad Sci U S A* **95**, 5539-44.
112. Kaspar, P., Dvorakova, M., Kralova, J., Pajer, P., Kozmik, Z. & Dvorak, M. (1999) Myb-interacting protein, ATBF1, represses transcriptional activity of Myb oncoprotein. *J Biol Chem* **274**, 14422-8.
113. Haas, M., Siegert, M., Schurmann, A., Sodeik, B. & Wolfes, H. (2004) c-Myb protein interacts with Rcd-1, a component of the CCR4 transcription mediator complex. *Biochemistry* **43**, 8152-9.
114. Allen, R.D., 3rd, Kim, H.K., Sarafova, S.D. & Siu, G. (2001) Negative regulation of CD4 gene expression by a HES-1-c-Myb complex. *Mol Cell Biol* **21**, 3071-82.
115. Kishi, H., Jin, Z.X., Wei, X.C., Nagata, T., Matsuda, T., Saito, S. & Muraguchi, A. (2002) Cooperative binding of c-Myb and Pax-5 activates the RAG-2 promoter in immature B cells. *Blood* **99**, 576-83.
116. Jin, Z.X., Kishi, H., Wei, X.C., Matsuda, T., Saito, S. & Muraguchi, A. (2002) Lymphoid enhancer-binding factor-1 binds and activates the recombination-activating gene-2 promoter together with c-Myb and Pax-5 in immature B cells. *J Immunol* **169**, 3783-92.
117. Dahle, O., Bakke, O. & Gabrielsen, O.S. (2004) c-Myb associates with PML in nuclear bodies in hematopoietic cells. *Exp Cell Res* **297**, 118-26.
118. Luscher, B., Christenson, E., Litchfield, D.W., Krebs, E.G. & Eisenman, R.N. (1990) Myb DNA binding inhibited by phosphorylation at a site deleted during oncogenic activation. *Nature* **344**, 517-22.
119. Oelgeschlager, M., Krieg, J., Luscher-Firzlaff, J.M. & Luscher, B. (1995) Casein kinase II phosphorylation site mutations in c-Myb affect DNA binding and transcriptional cooperativity with NF-M. *Mol Cell Biol* **15**, 5966-74.
120. Ramsay, R.G., Morrice, N., Van Eeden, P., Kanagasundaram, V., Nomura, T., De Blaquiere, J., Ishii, S. & Wettenhall, R. (1995) Regulation of c-Myb through protein phosphorylation and leucine zipper interactions. *Oncogene* **11**, 2113-20.
121. Bergholtz, S., Andersen, T.O., Andersson, K.B., Borrebaek, J., Luscher, B. & Gabrielsen, O.S. (2001) The highly conserved DNA-binding domains of A-, B- and c-Myb differ with respect to DNA-binding, phosphorylation and redox properties. *Nucleic Acids Res* **29**, 3546-56.
122. Andersson, K.B., Kowenz-Leutz, E., Brendeford, E.M., Tygsett, A.H., Leutz, A. & Gabrielsen, O.S. (2003) Phosphorylation-dependent Down-regulation of c-Myb DNA Binding Is Abrogated by a Point Mutation in the v-myb Oncogene. *J Biol Chem* **278**, 3816-24.
123. Bies, J., Feikova, S., Markus, J. & Wolff, L. (2001) Phosphorylation-Dependent Conformation and Proteolytic Stability of c-Myb. *Blood Cells Mol Dis* **27**, 422-8.
124. Bies, J. & Wolff, L. (1997) Oncogenic activation of c-Myb by carboxyl-terminal truncation leads to decreased proteolysis by the ubiquitin-26S proteasome pathway. *Oncogene* **14**, 203-12.
125. Feikova, S., Wolff, L. & Bies, J. (2000) Constitutive ubiquitination and degradation of c-myb by the 26S proteasome during proliferation and differentiation of myeloid cells. *Neoplasma* **47**, 212-8.
126. Bies, J., Nazarov, V. & Wolff, L. (1999) Identification of protein instability determinants in the carboxy- terminal region of c-Myb removed as a result of retroviral integration in murine monocytic leukemias. *J Virol* **73**, 2038-44.
127. Corradini, F., Cesi, V., Bartella, V., Pani, E., Bussolari, R., Candini, O. & Calabretta, B. (2005) Enhanced proliferative potential of hematopoietic cells expressing degradation-resistant c-Myb mutants. *J Biol Chem* **280**, 30254-62.
128. Kastan, M.B., Slamon, D.J. & Civin, C.I. (1989) Expression of protooncogene c-myb in normal human hematopoietic cells. *Blood* **73**, 1444-51.
129. Sitzmann, J., Noben-Trauth, K. & Klempnauer, K.H. (1995) Expression of mouse c-myb during embryonic development. *Oncogene* **11**, 2273-9.

130. Gonda, T.J. & Metcalf, D. (1984) Expression of myb, myc and fos proto-oncogenes during the differentiation of a murine myeloid leukaemia. *Nature* **310**, 249-51.
131. Westin, E.H., Gallo, R.C., Arya, S.K., Eva, A., Souza, L.M., Baluda, M.A., Aaronson, S.A. & Wong-Staal, F. (1982) Differential expression of the amv gene in human hematopoietic cells. *Proc Natl Acad Sci U S A* **79**, 2194-8.
132. Oh, I.H. & Reddy, E.P. (1999) The myb gene family in cell growth, differentiation and apoptosis. *Oncogene* **18**, 3017-33.
133. Ramsay, R.G. (2005) c-Myb a stem-progenitor cell regulator in multiple tissue compartments. *Growth Factors* **23**, 253-61.
134. Reilly, C.F., Kindy, M.S., Brown, K.E., Rosenberg, R.D. & Sonenshein, G.E. (1989) Heparin prevents vascular smooth muscle cell progression through the G1 phase of the cell cycle. *J Biol Chem* **264**, 6990-5.
135. Ess, K.C., Witte, D.P., Bascomb, C.P. & Aronow, B.J. (1999) Diverse developing mouse lineages exhibit high-level c-Myb expression in immature cells and loss of expression upon differentiation. *Oncogene* **18**, 1103-11.
136. Malaterre, J., Mantamadiotis, T., Dworkin, S., Lightowler, S., Yang, Q., Ransome, M.I., Turnley, A.M., Nichols, N.R., Emambokus, N.R., Frampton, J. & Ramsay, R.G. (2007) c-Myb is Required for Neural Progenitor Cell Proliferation and Maintenance of the Neural Stem Cell Niche in Adult Brain. *Stem Cells*.
137. Malaterre, J. et al. (2007) c-Myb is required for progenitor cell homeostasis in colonic crypts. *Proc Natl Acad Sci U S A* **104**, 3829-34.
138. Graf, T., von Kirchbach, A. & Beug, H. (1981) Characterization of the hematopoietic target cells of AEV, MC29 and AMV avian leukemia viruses. *Exp Cell Res* **131**, 331-43.
139. Radke, K., Beug, H., Kornfeld, S. & Graf, T. (1982) Transformation of both erythroid and myeloid cells by E26, an avian leukemia virus that contains the myb gene. *Cell* **31**, 643-53.
140. Clarke, M.F., Kukowska-Latallo, J.F., Westin, E., Smith, M. & Prochownik, E.V. (1988) Constitutive expression of a c-myb cDNA blocks Friend murine erythroleukemia cell differentiation. *Mol Cell Biol* **8**, 884-92.
141. Coll, J., Saule, S., Martin, P., Raes, M.B., Lagrou, C., Graf, T., Beug, H., Simon, I.E. & Stehelin, D. (1983) The cellular oncogenes c-myc, c-myb and c-erb are transcribed in defined types of avian hematopoietic cells. *Exp Cell Res* **149**, 151-62.
142. Craig, R.W. & Bloch, A. (1984) Early decline in c-myb oncogene expression in the differentiation of human myeloblastic leukemia (ML-1) cells induced with 12-O- tetradecanoylphorbol-13-acetate. *Cancer Res* **44**, 442-6.
143. Torelli, G., Selleri, L., Donelli, A., Ferrari, S., Emilia, G., Venturelli, D., Moretti, L. & Torelli, U. (1985) Activation of c-myb expression by phytohemagglutinin stimulation in normal human T lymphocytes. *Mol Cell Biol* **5**, 2874-7.
144. Schmidt, M., Nazarov, V., Stevens, L., Watson, R. & Wolff, L. (2000) Regulation of the resident chromosomal copy of c-myc by c-Myb is involved in myeloid leukemogenesis. *Mol Cell Biol* **20**, 1970-81.
145. Nakagoshi, H., Kanei-Ishii, C., Sawazaki, T., Mizuguchi, G. & Ishii, S. (1992) Transcriptional activation of the c-myc gene by the c-myb and B-myb gene products. *Oncogene* **7**, 1233-40.
146. Evans, J.L., Moore, T.L., Kuehl, W.M., Bender, T. & Ting, J.P. (1990) Functional analysis of c-Myb protein in T-lymphocytic cell lines shows that it trans-activates the c-myc promoter. *Mol Cell Biol* **10**, 5747-52.
147. Cogswell, J.P., Cogswell, P.C., Kuehl, W.M., Cuddihy, A.M., Bender, T.M., Engelke, U., Marcu, K.B. & Ting, J.P. (1993) Mechanism of c-myc regulation by c-Myb in different cell lineages. *Mol Cell Biol* **13**, 2858-69.
148. Wolff, L., Schmidt, M., Koller, R., Haviernik, P., Watson, R., Bies, J. & Maciag, K. (2001) Three Genes with Different Functions in Transformation are Regulated by c-Myb in Myeloid Cells. *Blood Cells Mol Dis* **27**, 483-8.
149. Brandt, T.L., Fraser, D.J., Leal, S., Halandras, P.M., Kroll, A.R. & Kroll, D.J. (1997) c-Myb trans-activates the human DNA topoisomerase IIalpha gene promoter. *J Biol Chem* **272**, 6278-84.
150. Miglarese, M.R., Halaban, R. & Gibson, N.W. (1997) Regulation of fibroblast growth factor 2 expression in melanoma cells by the c-MYB proto-oncoprotein. *Cell Growth Differ* **8**, 1199-210.
151. Hogg, A., Schirm, S., Nakagoshi, H., Bartley, P., Ishii, S., Bishop, J.M. & Gonda, T.J. (1997) Inactivation of a c-Myb/estrogen receptor fusion protein in transformed primary cells leads to granulocyte/macrophage differentiation and down regulation of c-kit but not c-myc or cdc2. *Oncogene* **15**, 2885-98.

152. Ratajczak, M.Z., Perrotti, D., Melotti, P., Powzaniuk, M., Calabretta, B., Onodera, K., Kregenow, D.A., Machalinski, B. & Gewirtz, A.M. (1998) Myb and ets proteins are candidate regulators of c-kit expression in human hematopoietic cells. *Blood* **91**, 1934-46.
153. Taylor, D., Badiani, P. & Weston, K. (1996) A dominant interfering Myb mutant causes apoptosis in T cells. *Genes Dev* **10**, 2732-44.
154. Frampton, J., Ramqvist, T. & Graf, T. (1996) v-Myb of E26 leukemia virus up-regulates bcl-2 and suppresses apoptosis in myeloid cells. *Genes Dev* **10**, 2720-31.
155. Thompson, M.A., Rosenthal, M.A., Ellis, S.L., Friend, A.J., Zorbas, M.I., Whitehead, R.H. & Ramsay, R.G. (1998) c-Myb down-regulation is associated with human colon cell differentiation, apoptosis, and decreased Bcl-2 expression. *Cancer Res* **58**, 5168-75.
156. Salomoni, P., Perrotti, D., Martinez, R., Franceschi, C. & Calabretta, B. (1997) Resistance to apoptosis in CTLL-2 cells constitutively expressing c-Myb is associated with induction of BCL-2 expression and Myb-dependent regulation of bcl-2 promoter activity. *Proc Natl Acad Sci U S A* **94**, 3296-301.
157. Selvakumaran, M., Lin, H.K., Sjin, R.T., Reed, J.C., Liebermann, D.A. & Hoffman, B. (1994) The novel primary response gene MyD118 and the proto-oncogenes myb, myc, and bcl-2 modulate transforming growth factor beta 1-induced apoptosis of myeloid leukemia cells. *Mol Cell Biol* **14**, 2352-60.
158. Bies, J., Mukhopadhyaya, R., Pierce, J. & Wolff, L. (1995) Only late, nonmitotic stages of granulocyte differentiation in 32Dcl3 cells are blocked by ectopic expression of murine c-myb and its truncated forms. *Cell Growth Differ* **6**, 59-68.
159. Sala, A., Casella, I., Grasso, L., Bellon, T., Reed, J.C., Miyashita, T. & Peschle, C. (1996) Apoptotic response to oncogenic stimuli: cooperative and antagonistic interactions between c-myb and the growth suppressor p53. *Cancer Res* **56**, 1991-6.
160. Mucenski, M.L., McLain, K., Kier, A.B., Swerdlow, S.H., Schreiner, C.M., Miller, T.A., Pietryga, D.W., Scott, W.J.J. & Potter, S.S. (1991) A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell* **65**, 677-89.
161. Thompson, M.A. & Ramsay, R.G. (1995) Myb: an old oncoprotein with new roles. *Bioessays* **17**, 341-50.
162. Jarvis, T.C., Alby, L.J., Beaudry, A.A., Wincott, F.E., Beigelman, L., McSwiggen, J.A., Usman, N. & Stinchcomb, D.T. (1996) Inhibition of vascular smooth muscle cell proliferation by ribozymes that cleave c-myb mRNA. *RNA* **2**, 419-28.
163. Opalinska, J.B., Kalota, A., Gifford, L.K., Lu, P., Jen, K.Y., Pradeepkumar, P.I., Barman, J., Kim, T.K., Swider, C.R., Chattopadhyaya, J. & Gewirtz, A.M. (2004) Oxetane modified, conformationally constrained, antisense oligodeoxyribonucleotides function efficiently as gene silencing molecules. *Nucleic Acids Res* **32**, 5791-9.
164. Emambokus, N., Vegiopoulos, A., Harman, B., Jenkinson, E., Anderson, G. & Frampton, J. (2003) Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. *EMBO J* **22**, 4478-88.
165. Sandberg, M.L., Sutton, S.E., Pletcher, M.T., Wiltshire, T., Tarantino, L.M., Hogenesch, J.B. & Cooke, M.P. (2005) c-Myb and p300 regulate hematopoietic stem cell proliferation and differentiation. *Dev Cell* **8**, 153-66.
166. Carpinelli, M.R. et al. (2004) Suppressor screen in Mpl-/- mice: c-Myb mutation causes supraphysiological production of platelets in the absence of thrombopoietin signaling. *Proc Natl Acad Sci U S A* **101**, 6553-8.
167. Ness, S.A., Marknell, A. & Graf, T. (1989) The v-myb oncogene product binds to and activates the promyelocyte- specific mim-1 gene. *Cell* **59**, 1115-25.
168. Allen, R.D., 3rd, Bender, T.P. & Siu, G. (1999) c-Myb is essential for early T cell development. *Genes Dev* **13**, 1073-8.
169. Badiani, P., Corbella, P., Kioussis, D., Marvel, J. & Weston, K. (1994) Dominant interfering alleles define a role for c-Myb in T-cell development. *Genes Dev* **8**, 770-82.
170. Pearson, R. & Weston, K. (2000) c-Myb regulates the proliferation of immature thymocytes following beta-selection. *Embo J* **19**, 6112-20.
171. Bender, T.P., Kremer, C.S., Kraus, M., Buch, T. & Rajewsky, K. (2004) Critical functions for c-Myb at three checkpoints during thymocyte development. *Nat Immunol* **5**, 721-9.
172. Nakayama, K., Yamamoto, R., Ishii, S. & Nakauchi, H. (1993) Binding of c-Myb to the core sequence of the CD4 promoter. *Int Immunol* **5**, 817-24.
173. Siu, G., Wurster, A.L., Lipsick, J.S. & Hedrick, S.M. (1992) Expression of the CD4 gene requires a Myb transcription factor. *Mol Cell Biol* **12**, 1592-604.

174. Hernandez-Munain, C. & Krangel, M.S. (1995) c-Myb and core-binding factor/PEBP2 display functional synergy but bind independently to adjacent sites in the T-cell receptor delta enhancer. *Mol Cell Biol* **15**, 3090-9.
175. Hernandez-Munain, C. & Krangel, M.S. (1994) Regulation of the T-cell receptor delta enhancer by functional cooperation between c-Myb and core-binding factors. *Mol Cell Biol* **14**, 473-83.
176. Wang, Q.F., Lauring, J. & Schlissel, M.S. (2000) c-Myb binds to a sequence in the proximal region of the RAG-2 promoter and is essential for promoter activity in T-lineage cells. *Mol Cell Biol* **20**, 9203-11.
177. Thomas, M.D., Kremer, C.S., Ravichandran, K.S., Rajewsky, K. & Bender, T.P. (2005) c-Myb is critical for B cell development and maintenance of follicular B cells. *Immunity* **23**, 275-86.
178. Rushton, J.J., Davis, L.M., Lei, W., Mo, X., Leutz, A. & Ness, S.A. (2003) Distinct changes in gene expression induced by A-Myb, B-Myb and c-Myb proteins. *Oncogene* **22**, 308-13.
179. Liu, F., Lei, W., O'Rourke, J.P. & Ness, S.A. (2006) Oncogenic mutations cause dramatic, qualitative changes in the transcriptional activity of c-Myb. *Oncogene* **25**, 795-805.
180. Lei, W., Rushton, J.J., Davis, L.M., Liu, F. & Ness, S.A. (2004) Positive and negative determinants of target gene specificity in myb transcription factors. *J Biol Chem* **279**, 29519-27.
181. Lang, G., White, J.R., Argent-Katwala, M.J., Allinson, C.G. & Weston, K. (2005) Myb proteins regulate the expression of diverse target genes. *Oncogene* **24**, 1375-84.
182. Burk, O., Worpenberg, S., Haenig, B. & Klempnauer, K.H. (1997) tom-1, a novel v-Myb target gene expressed in AMV- and E26-transformed myelomonocytic cells. *EMBO J* **16**, 1371-80.
183. Burk, O. & Klempnauer, K.H. (1999) Myb and Ets transcription factors cooperate at the myb-inducible promoter of the tom-1 gene. *Biochim Biophys Acta* **1446**, 243-52.
184. Burk, O. & Klempnauer, K.H. (1991) Estrogen-dependent alterations in differentiation state of myeloid cells caused by a v-myb/estrogen receptor fusion protein. *EMBO J* **10**, 3713-9.
185. Worpenberg, S., Burk, O. & Klempnauer, K.H. (1997) The chicken adenosine receptor 2B gene is regulated by v-myb. *Oncogene* **15**, 213-21.
186. Gundelach, H., Braas, D. & Klempnauer, K.H. (2007) The promoter regions of the Myb-regulated Adora2B and Mcm4 genes co-localize with origins of DNA replication. *BMC Mol Biol* **8**, 75.
187. Mink, S., Jaswal, S., Burk, O. & Klempnauer, K.H. (1999) The v-Myb oncoprotein activates C/EBPbeta expression by stimulating an autoregulatory loop at the C/EBPbeta promoter. *Biochim Biophys Acta* **1447**, 175-84.
188. Kintscher, J., Yamkamon, V., Braas, D. & Klempnauer, K.H. (2004) Identification of a Myb-responsive enhancer of the chicken C/EBPbeta gene. *Oncogene* **23**, 5807-14.
189. Braas, D., Gundelach, H. & Klempnauer, K.H. (2004) The glioma-amplified sequence 41 gene (GAS41) is a direct Myb target gene. *Nucleic Acids Res* **32**, 4750-7.
190. Schlichter, U., Burk, O., Worpenberg, S. & Klempnauer, K.H. (2001) The chicken Pdcd4 gene is regulated by v-Myb. *Oncogene* **20**, 231-9.
191. Schlichter, U., Kattmann, D., Appl, H., Miethe, J., Brehmer-Fastnacht, A. & Klempnauer, K.H. (2001) Identification of the myb-inducible promoter of the chicken Pdcd4 gene. *Biochim Biophys Acta* **1520**, 99-104.
192. Melotti, P. & Calabretta, B. (1994) Ets-2 and c-Myb act independently in regulating expression of the hematopoietic stem cell antigen CD34. *J Biol Chem* **269**, 25303-9.
193. Melotti, P., Ku, D.H. & Calabretta, B. (1994) Regulation of the expression of the hematopoietic stem cell antigen CD34: role of c-myb. *J Exp Med* **179**, 1023-8.
194. Luchetti, M.M., Paroncini, P., Majlingova, P., Frampton, J., Mucenski, M., Baroni, S.S., Sambo, P., Golay, J., Introna, M. & Gabrielli, A. (2003) Characterization of the c-Myb-responsive region and regulation of the human type I collagen alpha 2 chain gene by c-Myb. *J Biol Chem* **278**, 1533-41.
195. Kowenz-Leutz, E., Herr, P., Niss, K. & Leutz, A. (1997) The homeobox gene GBX2, a target of the myb oncogene, mediates autocrine growth and monocyte differentiation. *Cell* **91**, 185-95.
196. Shetzline, S.E., Rallapalli, R., Dowd, K.J., Zou, S., Nakata, Y., Swider, C.R., Kalota, A., Choi, J.K. & Gewirtz, A.M. (2004) Neuromedin U: a Myb-regulated autocrine growth factor for human myeloid leukemias. *Blood* **104**, 1833-40.
197. Chen, J., Kremer, C.S. & Bender, T.P. (2006) The carbonic anhydrase I locus contains a c-Myb target promoter and modulates differentiation of murine erythroleukemia cells. *Oncogene* **25**, 2758-72.
198. Maurice, D., Hooper, J., Lang, G. & Weston, K. (2007) c-Myb regulates lineage choice in developing thymocytes via its target gene Gata3. *Embo J* **26**, 3629-40.
199. Hooper, J., Maurice, D., Argent-Katwala, M.J. & Weston, K. (2007) Myb proteins regulate expression of histone variant H2A.Z during thymocyte development. *Immunology*.
200. Reiss, K., Ferber, A., Travali, S., Porcu, P., Phillips, P.D. & Baserga, R. (1991) The protooncogene c-myb increases the expression of insulin-like growth factor 1 and insulin-like growth factor 1 receptor messenger RNAs by a transcriptional mechanism. *Cancer Res* **51**, 5997-6000.

201. Tanno, B., Negroni, A., Vitali, R., Pirozzoli, M.C., Cesi, V., Mancini, C., Calabretta, B. & Raschella, G. (2002) Expression of insulin-like growth factor-binding protein 5 in neuroblastoma cells is regulated at the transcriptional level by c-Myb and B-Myb via direct and indirect mechanisms. *J Biol Chem* **277**, 23172-80.
202. Britos-Bray, M. & Friedman, A.D. (1997) Core binding factor cannot synergistically activate the myeloperoxidase proximal enhancer in immature myeloid cells without c-Myb. *Mol Cell Biol* **17**, 5127-35.
203. Bartley, P.A., Lutwyche, J.K. & Gonda, T.J. (2001) Identification and validation of candidate myb target genes. *Blood Cells Mol Dis* **27**, 409-15.
204. Ess, K.C., Whitaker, T.L., Cost, G.J., Witte, D.P., Hutton, J.J. & Aronow, B.J. (1995) A central role for a single c-Myb binding site in a thymic locus control region. *Mol Cell Biol* **15**, 5707-15.
205. Shapiro, L.H. (1995) Myb and Ets proteins cooperate to transactivate an early myeloid gene. *J Biol Chem* **270**, 8763-71.
206. Ku, D.H., Wen, S.C., Engelhard, A., Nicolaides, N.C., Lipson, K.E., Marino, T.A. & Calabretta, B. (1993) c-myb transactivates cdc2 expression via Myb binding sites in the 5'- flanking region of the human cdc2 gene. *J Biol Chem* **268**, 2255-9.
207. Nicolaides, N.C., Gualdi, R., Casadevall, C., Manzella, L. & Calabretta, B. (1991) Positive autoregulation of c-myb expression via Myb binding sites in the 5' flanking region of the human c-myb gene. *Mol Cell Biol* **11**, 6166-76.
208. Ramsay, R.G., Friend, A., Vizantios, Y., Freeman, R., Sicurella, C., Hammett, F., Armes, J. & Venter, D. (2000) Cyclooxygenase-2, a colorectal cancer nonsteroidal anti-inflammatory drug target, is regulated by c-MYB. *Cancer Res* **60**, 1805-9.
209. Muller, C., Yang, R., Idos, G., Tidow, N., Diederichs, S., Koch, O.M., Verbeek, W., Bender, T.P. & Koeffler, H.P. (1999) c-myb transactivates the human cyclin A1 promoter and induces cyclin A1 gene expression. *Blood* **94**, 4255-62.
210. Robert, I., Sutter, A. & Quirin-Stricker, C. (2002) Synergistic activation of the human choline acetyltransferase gene by c- Myb and C/EBPbeta. *Brain Res Mol Brain Res* **106**, 124.
211. McCracken, S., Leung, S., Bosselut, R., Ghysdael, J. & Miyamoto, N.G. (1994) Myb and Ets related transcription factors are required for activity of the human lck type I promoter. *Oncogene* **9**, 3609-15.
212. Yang, H., Huang, Z.Z., Wang, J. & Lu, S.C. (2001) The role of c-Myb and Sp1 in the up-regulation of methionine adenosyltransferase 2A gene expression in human hepatocellular carcinoma. *FASEB J* **15**, 1507-16.
213. Lutz, P.G., Houzel-Charavel, A., Moog-Lutz, C. & Cayre, Y.E. (2001) Myeloblastin is an Myb target gene: mechanisms of regulation in myeloid leukemia cells growth-arrested by retinoic acid. *Blood* **97**, 2449-56.
214. Sicurella, C., Freeman, R., Micallef, S., Mucenski, M.L., Bertoncello, I. & Ramsay, R.G. (2001) Defective Stem Cell Factor Expression in c-myb Null Fetal Liver Stroma. *Blood Cells Mol Dis* **27**, 470-8.
215. Hsiang, Y.H., Goldman, J.P. & Raulet, D.H. (1995) The role of c-Myb or a related factor in regulating the T cell receptor gamma gene enhancer. *J Immunol* **154**, 5195-204.
216. Matre, V., Hovring, P.I., Fjeldheim, A.K., Helgeland, L., Orvain, C., Andersson, K.B., Gautvik, K.M. & Gabrielsen, O.S. (2003) The human neuroendocrine thyrotropin-releasing hormone receptor promoter is activated by the haematopoietic transcription factor c-Myb. *Biochem J* **372**, 851-9.
217. Zhang, X., Xing, G., Fraizer, G.C. & Saunders, G.F. (1997) Transactivation of an intronic hematopoietic-specific enhancer of the human Wilms' tumor 1 gene by GATA-1 and c-Myb. *J Biol Chem* **272**, 29272-80.
218. Aurigemma, R.E., Blair, D.G. & Ruscetti, S.K. (1992) Transactivation of erythroid transcription factor GATA-1 by a myb-ets- containing retrovirus. *J Virol* **66**, 3056-61.
219. Nuchprayoon, I., Meyers, S., Scott, L.M., Suzow, J., Hiebert, S. & Friedman, A.D. (1994) PEBP2/CBF, the murine homolog of the human myeloid AML1 and PEBP2 beta/CBF beta proto-oncoproteins, regulates the murine myeloperoxidase and neutrophil elastase genes in immature myeloid cells. *Mol Cell Biol* **14**, 5558-68.
220. Oelgeschlager, M., Nuchprayoon, I., Luscher, B. & Friedman, A.D. (1996) C/EBP, c-Myb, and PU.1 cooperate to regulate the neutrophil elastase promoter. *Mol Cell Biol* **16**, 4717-25.
221. Plaza, S., Turque, N., Dozier, C., Bailly, M. & Saule, S. (1995) C-Myb acts as transcriptional activator of the quail PAX6 (PAX-QNR) promoter through two different mechanisms. *Oncogene* **10**, 329-40.
222. Reddy, M.A., Yang, B.S., Yue, X., Barnett, C.J., Ross, I.L., Sweet, M.J., Hume, D.A. & Ostrowski, M.C. (1994) Opposing actions of c-ets/PU.1 and c-myb protooncogene products in regulating the macrophage-specific promoters of the human and mouse colony-stimulating factor-1 receptor (c-fms) genes. *J Exp Med* **180**, 2309-19.

223. Mizuguchi, G., Kanei-Ishii, C., Takahashi, T., Yasukawa, T., Nagase, T., Horikoshi, M., Yamamoto, T. & Ishii, S. (1995) c-Myb repression of c-erbB-2 transcription by direct binding to the c- erbB-2 promoter. *J Biol Chem* **270**, 9384-9.
224. Ganter, B. & Lipsick, J.S. (1997) Myb binding sites within the N-ras promoter repress transcription. *Oncogene* **15**, 193-202.
225. Monticelli, S., Ghittoni, R., Kabesch, M. & Vercelli, D. (2002) Myb proteins repress human Ig varepsilon germline transcription by inhibiting STAT6-dependent promoter activation. *Mol Immunol* **38**, 1129-38.
226. Berge, T., Matre, V., Brendeford, E.M., Saether, T., Luscher, B. & Gabrielsen, O.S. (2007) Revisiting a selection of target genes for the hematopoietic transcription factor c-Myb using chromatin immunoprecipitation and c-Myb knockdown. *Blood Cells Mol Dis* **39**, 278-286.
227. Bischoff, K.M., Pishko, E.J., Genovese, K.J., Crippen, T.L., Holtzapple, C.K., Stanker, L.H., Nisbet, D.J. & Kogut, M.H. (2001) Chicken mim-1 protein, P33, is a heterophil chemotactic factor present in Salmonella enteritidis immune lymphokine. *J Food Prot* **64**, 1503-9.
228. Allen, S.C. & Hebbes, T.R. (2003) Myb induced myeloid protein 1 (Mim-1) is an acetyltransferase. *FEBS Lett* **534**, 119-24.
229. Zobel, A., Kalkbrenner, F., Guehmann, S., Nawrath, M., Vorbrueggen, G. & Moelling, K. (1991) Interaction of the v-and c-Myb proteins with regulatory sequences of the human c-myc gene. *Oncogene* **6**, 1397-407.
230. Zobel, A., Kalkbrenner, F., Vorbrueggen, G. & Moelling, K. (1992) Transactivation of the human c-myc gene by c-Myb. *Biochem Biophys Res Commun* **186**, 715-22.
231. Appl, H. & Klempnauer, K.H. (2002) Targeted disruption of c-myb in the chicken pre B-cell line DT40. *Oncogene* **21**, 3076-81.
232. Guerra, J., Withers, D.A. & Boxer, L.M. (1995) Myb binding sites mediate negative regulation of c-myb expression in T- cell lines. *Blood* **86**, 1873-80.
233. Alitalo, K., Winqvist, R., Lin, C.C., de la Chapelle, A., Schwab, M. & Bishop, J.M. (1984) Aberrant expression of an amplified c-myb oncogene in two cell lines from a colon carcinoma. *Proc Natl Acad Sci U S A* **81**, 4534-8.
234. Ramsay, R.G., Barton, A.L. & Gonda, T.J. (2003) Targeting c-Myb expression in human disease. *Expert Opin Ther Targets* **7**, 235-48.
235. Guerin, M., Sheng, Z.M., Andrieu, N. & Riou, G. (1990) Strong association between c-myb and oestrogen-receptor expression in human breast cancer. *Oncogene* **5**, 131-5.
236. Kauraniemi, P., Hedenfalk, I., Persson, K., Duggan, D.J., Tanner, M., Johannsson, O., Olsson, H., Trent, J.M., Isola, J. & Borg, A. (2000) MYB oncogene amplification in hereditary BRCA1 breast cancer. *Cancer Res* **60**, 5323-8.
237. Ramsay, R.G., Thompson, M.A., Hayman, J.A., Reid, G., Gonda, T.J. & Whitehead, R.H. (1992) Myb expression is higher in malignant human colonic carcinoma and premalignant adenomatous polyps than in normal mucosa. *Cell Growth Differ* **3**, 723-30.
238. Alexander, R.J., Buxbaum, J.N. & Raicht, R.F. (1991) Enhanced expression of oncogene-encoded mRNA in a rat model of colon cancer. *Am J Med Sci* **301**, 238-45.
239. Alexander, R.J., Panja, A., Kaplan-Liss, E., Mayer, L. & Raicht, R.F. (1996) Expression of protooncogene-encoded mRNA by colonic epithelial cells in inflammatory bowel disease. *Dig Dis Sci* **41**, 660-9.
240. Brabender, J. et al. (2001) Increased c-myb mRNA expression in Barrett's esophagus and Barrett's-associated adenocarcinoma. *J Surg Res* **99**, 301-6.
241. Su, A.I., Welsh, J.B., Sapinoso, L.M., Kern, S.G., Dimitrov, P., Lapp, H., Schultz, P.G., Powell, S.M., Moskaluk, C.A., Frierson, H.F., Jr. & Hampton, G.M. (2001) Molecular classification of human carcinomas by use of gene expression signatures. *Cancer Res* **61**, 7388-93.
242. Sinclair, P., Harrison, C.J., Jarosova, M. & Foroni, L. (2005) Analysis of balanced rearrangements of chromosome 6 in acute leukemia: clustered breakpoints in q22-q23 and possible involvement of c-MYB in a new recurrent translocation, t(6;7)(q23;q32 through 36). *Haematologica* **90**, 602-11.
243. Clappier, E. et al. (2007) The C-MYB locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children. *Blood* **110**, 1251-61.
244. Lahortiga, I. et al. (2007) Duplication of the MYB oncogene in T cell acute lymphoblastic leukemia. *Nat Genet* **39**, 593-5.
245. Drabsch, Y., Hugo, H., Zhang, R., Dowhan, D.H., Miao, Y.R., Gewirtz, A.M., Barry, S.C., Ramsay, R.G. & Gonda, T.J. (2007) Mechanism of and requirement for estrogen-regulated MYB expression in estrogen-receptor-positive breast cancer cells. *Proc Natl Acad Sci U S A* **104**, 13762-7.

246. Mahajan, R., Delphin, C., Guan, T., Gerace, L. & Melchior, F. (1997) A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* **88**, 97-107.
247. Matunis, M.J., Coutavas, E. & Blobel, G. (1996) A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol* **135**, 1457-70.
248. Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M. & Ahringer, J. (2000) Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. *Nature* **408**, 325-30.
249. Hayashi, T., Seki, M., Maeda, D., Wang, W., Kawabe, Y., Seki, T., Saitoh, H., Fukagawa, T., Yagi, H. & Enomoto, T. (2002) Ubc9 is essential for viability of higher eukaryotic cells. *Exp Cell Res* **280**, 212-21.
250. Nacerddine, K., Lehembre, F., Bhaumik, M., Artus, J., Cohen-Tannoudji, M., Babinet, C., Pandolfi, P.P. & Dejean, A. (2005) The SUMO pathway is essential for nuclear integrity and chromosome segregation in mice. *Dev Cell* **9**, 769-79.
251. Hoeller, D., Hecker, C.M. & Dikic, I. (2006) Ubiquitin and ubiquitin-like proteins in cancer pathogenesis. *Nat Rev Cancer* **6**, 776-88.
252. Bayer, P., Arndt, A., Metzger, S., Mahajan, R., Melchior, F., Jaenicke, R. & Becker, J. (1998) Structure determination of the small ubiquitin-related modifier SUMO-1. *J Mol Biol* **280**, 275-86.
253. Gill, G. (2004) SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev* **18**, 2046-59.
254. Kerscher, O., Felberbaum, R. & Hochstrasser, M. (2006) Modification of Proteins by Ubiquitin and Ubiquitin-Like Proteins. *Annu Rev Cell Dev Biol*.
255. Hochstrasser, M. (2000) Evolution and function of ubiquitin-like protein-conjugation systems. *Nat Cell Biol* **2**, E153-7.
256. Ciechanover, A. & Ben-Saadon, R. (2004) N-terminal ubiquitination: more protein substrates join in. *Trends Cell Biol* **14**, 103-6.
257. Schmidt, M.H. & Dikic, I. (2006) Ubiquitin and NEDD8: brothers in arms. *Sci STKE* **2006**, pe50.
258. Gill, G. (2005) Something about SUMO inhibits transcription. *Curr Opin Genet Dev* **15**, 536-41.
259. Hay, R.T. (2005) SUMO: a history of modification. *Mol Cell* **18**, 1-12.
260. Hicke, L., Schubert, H.L. & Hill, C.P. (2005) Ubiquitin-binding domains. *Nat Rev Mol Cell Biol* **6**, 610-21.
261. Kerscher, O. (2007) SUMO junction-what's your function? New insights through SUMO-interacting motifs. *EMBO Rep* **8**, 550-5.
262. Kirkin, V. & Dikic, I. (2007) Role of ubiquitin- and Ubl-binding proteins in cell signaling. *Curr Opin Cell Biol* **19**, 199-205.
263. Bohren, K.M., Nadkarni, V., Song, J.H., Gabbay, K.H. & Owerbach, D. (2004) A M55V polymorphism in a novel SUMO gene (SUMO-4) differentially activates heat shock transcription factors and is associated with susceptibility to type I diabetes mellitus. *J Biol Chem* **279**, 27233-8.
264. Saitoh, H. & Hinchey, J. (2000) Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J Biol Chem* **275**, 6252-8.
265. Tatham, M.H., Jaffray, E., Vaughan, O.A., Desterro, J.M., Botting, C.H., Naismith, J.H. & Hay, R.T. (2001) Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem* **276**, 35368-74.
266. Owerbach, D., McKay, E.M., Yeh, E.T., Gabbay, K.H. & Bohren, K.M. (2005) A proline-90 residue unique to SUMO-4 prevents maturation and sumoylation. *Biochem Biophys Res Commun* **337**, 517-20.
267. Su, A.I. et al. (2002) Large-scale analysis of the human and mouse transcriptomes. *Proc Natl Acad Sci U S A* **99**, 4465-70.
268. Johnson, E.S. & Gupta, A.A. (2001) An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell* **106**, 735-44.
269. Pichler, A., Gast, A., Seeler, J.S., Dejean, A. & Melchior, F. (2002) The nucleoporin RanBP2 has SUMO1 E3 ligase activity. *Cell* **108**, 109-20.
270. Vertegaal, A.C. (2007) Small ubiquitin-related modifiers in chains. *Biochem Soc Trans* **35**, 1422-3.
271. Capili, A.D. & Lima, C.D. (2007) Structure and analysis of a complex between SUMO and Ubc9 illustrates features of a conserved E2-Ubl interaction. *J Mol Biol* **369**, 608-18.
272. Gong, L., Kamitani, T., Fujise, K., Caskey, L.S. & Yeh, E.T. (1997) Preferential interaction of sentrin with a ubiquitin-conjugating enzyme, Ubc9. *J Biol Chem* **272**, 28198-201.
273. Song, J., Durrin, L.K., Wilkinson, T.A., Krontiris, T.G. & Chen, Y. (2004) Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *Proc Natl Acad Sci U S A* **101**, 14373-8.
274. Song, J., Zhang, Z., Hu, W. & Chen, Y. (2005) Small ubiquitin-like modifier (SUMO) recognition of a SUMO binding motif: a reversal of the bound orientation. *J Biol Chem* **280**, 40122-9.

275. Reverter, D. & Lima, C.D. (2005) Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. *Nature* **435**, 687-92.
276. Hecker, C.M., Rabiller, M., Haglund, K., Bayer, P. & Dikic, I. (2006) Specification of SUMO1- and SUMO2-interacting motifs. *J Biol Chem* **281**, 16117-27.
277. Tatham, M.H., Kim, S., Jaffray, E., Song, J., Chen, Y. & Hay, R.T. (2005) Unique binding interactions among Ubc9, SUMO and RanBP2 reveal a mechanism for SUMO paralog selection. *Nat Struct Mol Biol* **12**, 67-74.
278. Sampson, D.A., Wang, M. & Matunis, M.J. (2001) The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification. *J Biol Chem* **276**, 21664-9.
279. Rodriguez, M.S., Dargemont, C. & Hay, R.T. (2001) SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J Biol Chem* **276**, 12654-9.
280. Johnson, E.S. & Blobel, G. (1999) Cell cycle-regulated attachment of the ubiquitin-related protein SUMO to the yeast septins. *J Cell Biol* **147**, 981-94.
281. Bernier-Villamor, V., Sampson, D.A., Matunis, M.J. & Lima, C.D. (2002) Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. *Cell* **108**, 345-56.
282. Yang, S.H., Galanis, A., Witty, J. & Sharrocks, A.D. (2006) An extended consensus motif enhances the specificity of substrate modification by SUMO. *EMBO J* **25**, 5083-93.
283. Hietakangas, V., Anckar, J., Blomster, H.A., Fujimoto, M., Palvimo, J.J., Nakai, A. & Sistonen, L. (2006) PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc Natl Acad Sci U S A* **103**, 45-50.
284. Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G. & Jentsch, S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419**, 135-41.
285. Xirodimas, D.P., Chisholm, J., Desterro, J.M., Lane, D.P. & Hay, R.T. (2002) P14ARF promotes accumulation of SUMO-1 conjugated (H)Mdm2. *FEBS Lett* **528**, 207-11.
286. Miyauchi, Y., Yogosawa, S., Honda, R., Nishida, T. & Yasuda, H. (2002) Sumoylation of Mdm2 by protein inhibitor of activated STAT (PIAS) and RanBP2 enzymes. *J Biol Chem* **277**, 50131-6.
287. Comerford, K.M., Leonard, M.O., Karhausen, J., Carey, R., Colgan, S.P. & Taylor, C.T. (2003) Small ubiquitin-related modifier-1 modification mediates resolution of CREB-dependent responses to hypoxia. *Proc Natl Acad Sci U S A* **100**, 986-91.
288. Hay, R.T. (2007) SUMO-specific proteases: a twist in the tail. *Trends Cell Biol* **17**, 370-6.
289. Mukhopadhyay, D. & Dasso, M. (2007) Modification in reverse: the SUMO proteases. *Trends Biochem Sci* **32**, 286-95.
290. Shen, L.N., Dong, C., Liu, H., Naismith, J.H. & Hay, R.T. (2006) The structure of SENP1-SUMO-2 complex suggests a structural basis for discrimination between SUMO paralogues during processing. *Biochem J* **397**, 279-88.
291. Xu, Z. & Au, S.W. (2005) Mapping residues of SUMO precursors essential in differential maturation by SUMO-specific protease, SENP1. *Biochem J* **386**, 325-30.
292. Reverter, D. & Lima, C.D. (2004) A basis for SUMO protease specificity provided by analysis of human Senp2 and a Senp2-SUMO complex. *Structure (Camb)* **12**, 1519-31.
293. Gong, L., Li, B., Millas, S. & Yeh, E.T. (1999) Molecular cloning and characterization of human AOS1 and UBA2, components of the sentrin-activating enzyme complex. *FEBS Lett* **448**, 185-9.
294. Okuma, T., Honda, R., Ichikawa, G., Tsumagari, N. & Yasuda, H. (1999) In vitro SUMO-1 modification requires two enzymatic steps, E1 and E2. *Biochem Biophys Res Commun* **254**, 693-8.
295. Boggio, R., Colombo, R., Hay, R.T., Draetta, G.F. & Chiocca, S. (2004) A mechanism for inhibiting the SUMO pathway. *Mol Cell* **16**, 549-61.
296. Desterro, J.M., Thomson, J. & Hay, R.T. (1997) Ubch9 conjugates SUMO but not ubiquitin. *FEBS Lett* **417**, 297-300.
297. Johnson, E.S. & Blobel, G. (1997) Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. *J Biol Chem* **272**, 26799-802.
298. Tatham, M.H., Kim, S., Yu, B., Jaffray, E., Song, J., Zheng, J., Rodriguez, M.S., Hay, R.T. & Chen, Y. (2003) Role of an N-terminal site of Ubc9 in SUMO-1, -2, and -3 binding and conjugation. *Biochemistry* **42**, 9959-69.
299. Kuehn, M.R. (2005) Mouse Ubc9 knockout: many path(way)s to ruin. *Dev Cell* **9**, 727-8.
300. Tatham, M.H., Chen, Y. & Hay, R.T. (2003) Role of two residues proximal to the active site of Ubc9 in substrate recognition by the Ubc9.SUMO-1 thiolester complex. *Biochemistry* **42**, 3168-79.
301. Melchior, F., Schergaut, M. & Pichler, A. (2003) SUMO: ligases, isopeptidases and nuclear pores. *Trends Biochem Sci* **28**, 612-8.

302. Kagey, M.H., Melhuish, T.A. & Wotton, D. (2003) The polycomb protein Pc2 is a SUMO E3. *Cell* **113**, 127-37.
303. Kahyo, T., Nishida, T. & Yasuda, H. (2001) Involvement of PIAS1 in the sumoylation of tumor suppressor p53. *Mol Cell* **8**, 713-8.
304. Chung, C.D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P. & Shuai, K. (1997) Specific inhibition of Stat3 signal transduction by PIAS3. *Science* **278**, 1803-5.
305. Liu, B., Liao, J., Rao, X., Kushner, S.A., Chung, C.D., Chang, D.D. & Shuai, K. (1998) Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci U S A* **95**, 10626-31.
306. Shuai, K. (2000) Modulation of STAT signaling by STAT-interacting proteins. *Oncogene* **19**, 2638-44.
307. Hochstrasser, M. (2001) SP-RING for SUMO: new functions bloom for a ubiquitin-like protein. *Cell* **107**, 5-8.
308. Liu, B., Mink, S., Wong, K.A., Stein, N., Getman, C., Dempsey, P.W., Wu, H. & Shuai, K. (2004) PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. *Nat Immunol* **5**, 891-8.
309. Roth, W., Sustmann, C., Kieslinger, M., Gilmozzi, A., Irmer, D., Kremmer, E., Turck, C. & Grosschedl, R. (2004) PIASy-deficient mice display modest defects in IFN and Wnt signaling. *J Immunol* **173**, 6189-99.
310. Yang, S.H. & Sharrocks, A.D. (2005) PIASx acts as an Elk-1 coactivator by facilitating derepression. *EMBO J* **24**, 2161-71.
311. Gross, M., Yang, R., Top, I., Gasper, C. & Shuai, K. (2004) PIASy-mediated repression of the androgen receptor is independent of sumoylation. *Oncogene* **23**, 3059-66.
312. Kim, J., Sharma, S., Li, Y., Cobos, E., Palvimo, J.J. & Williams, S.C. (2005) Repression and Coactivation of CCAAT/Enhancer-binding Protein epsilon by Sumoylation and Protein Inhibitor of Activated STATx Proteins. *J Biol Chem* **280**, 12246-54.
313. Kirsh, O., Seeler, J.S., Pichler, A., Gast, A., Muller, S., Miska, E., Mathieu, M., Harel-Bellan, A., Kouzarides, T., Melchior, F. & Dejean, A. (2002) The SUMO E3 ligase RanBP2 promotes modification of the HDAC4 deacetylase. *EMBO J* **21**, 2682-91.
314. Saitoh, H., Pu, R., Cavenagh, M. & Dasso, M. (1997) RanBP2 associates with Ubc9p and a modified form of RanGAP1. *Proc Natl Acad Sci U S A* **94**, 3736-41.
315. Matunis, M.J., Wu, J. & Blobel, G. (1998) SUMO-1 modification and its role in targeting the Ran GTPase-activating protein, RanGAP1, to the nuclear pore complex. *J Cell Biol* **140**, 499-509.
316. Kagey, M.H., Melhuish, T.A., Powers, S.E. & Wotton, D. (2005) Multiple activities contribute to Pc2 E3 function. *EMBO J* **24**, 108-19.
317. Calzado, M.A., Renner, F., Roscic, A. & Schmitz, M.L. (2007) HIPK2: a versatile switchboard regulating the transcription machinery and cell death. *Cell Cycle* **6**, 139-43.
318. Lin, X., Sun, B., Liang, M., Liang, Y.Y., Gast, A., Hildebrand, J., Brunicardi, F.C., Melchior, F. & Feng, X.H. (2003) Opposed regulation of corepressor CtBP by SUMOylation and PDZ binding. *Mol Cell* **11**, 1389-96.
319. Endter, C., Kzhyshkowska, J., Stauber, R. & Dobner, T. (2001) SUMO-1 modification required for transformation by adenovirus type 5 early region 1B 55-kDa oncoprotein. *Proc Natl Acad Sci U S A* **98**, 11312-7.
320. Huang, T.T., Wuerzberger-Davis, S.M., Wu, Z.H. & Miyamoto, S. (2003) Sequential modification of NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress. *Cell* **115**, 565-76.
321. Chen, A., Wang, P.Y., Yang, Y.C., Huang, Y.H., Yeh, J.J., Chou, Y.H., Cheng, J.T., Hong, Y.R. & Li, S.S. (2006) SUMO regulates the cytoplasmonuclear transport of its target protein Daxx. *J Cell Biochem* **98**, 895-911.
322. Pichler, A. & Melchior, F. (2002) Ubiquitin-related modifier SUMO1 and nucleocytoplasmic transport. *Traffic* **3**, 381-7.
323. Muller, S., Ledl, A. & Schmidt, D. (2004) SUMO: a regulator of gene expression and genome integrity. *Oncogene* **23**, 1998-2008.
324. Hardeland, U., Steinacher, R., Jiricny, J. & Schar, P. (2002) Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover. *EMBO J* **21**, 1456-64.
325. Takahashi, H., Hatakeyama, S., Saitoh, H. & Nakayama, K.I. (2005) Noncovalent SUMO-1 binding activity of thymine DNA glycosylase (TDG) is required for its SUMO-1 modification and colocalization with the promyelocytic leukemia protein. *J Biol Chem* **280**, 5611-21.
326. Mohan, R.D., Rao, A., Gagliardi, J. & Tini, M. (2007) SUMO-1-dependent allosteric regulation of thymine DNA glycosylase alters subnuclear localization and CBP/p300 recruitment. *Mol Cell Biol* **27**, 229-43.

327. Seeler, J.S. & Dejean, A. (2003) Nuclear and unclear functions of SUMO. *Nat Rev Mol Cell Biol* **4**, 690-9.
328. Everett, R.D., Lomonte, P., Sternsdorf, T., van Driel, R. & Orr, A. (1999) Cell cycle regulation of PML modification and ND10 composition. *J Cell Sci* **112 (Pt 24)**, 4581-8.
329. Desterro, J.M., Rodriguez, M.S. & Hay, R.T. (1998) SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. *Mol Cell* **2**, 233-9.
330. Ross, S., Best, J.L., Zon, L.I. & Gill, G. (2002) SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol Cell* **10**, 831-42.
331. Shiio, Y. & Eisenman, R.N. (2003) From The Cover: Histone sumoylation is associated with transcriptional repression. *Proc Natl Acad Sci U S A* **100**, 13225-30.
332. Girdwood, D., Bumpass, D., Vaughan, O.A., Thain, A., Anderson, L.A., Snowden, A.W., Garcia-Wilson, E., Perkins, N.D. & Hay, R.T. (2003) P300 transcriptional repression is mediated by SUMO modification. *Mol Cell* **11**, 1043-54.
333. David, G., Neptune, M.A. & DePinho, R.A. (2002) SUMO-1 modification of histone deacetylase 1 (HDAC1) modulates its biological activities. *J Biol Chem* **277**, 23658-63.
334. Boyer-Guittaut, M., Birsoy, K., Potel, C., Elliott, G., Jaffray, E., Desterro, J.M., Hay, R.T. & Oelgeschlager, T. (2005) SUMO-1 modification of human transcription factor (TF) IID complex subunits: inhibition of TFIID promoter-binding activity through SUMO-1 modification of hsTAF5. *J Biol Chem* **280**, 9937-45.
335. Yang, S.H. & Sharrocks, A.D. (2004) SUMO promotes HDAC-mediated transcriptional repression. *Mol Cell* **13**, 611-7.
336. Eaton, E.M. & Sealy, L. (2003) Modification of CCAAT/Enhancer-binding Protein-{beta} by the Small Ubiquitin-like Modifier (SUMO) Family Members, SUMO-2 and SUMO-3. *J Biol Chem* **278**, 33416-33421.
337. Poukka, H., Karvonen, U., Janne, O.A. & Palvimo, J.J. (2000) Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). *Proc Natl Acad Sci U S A* **97**, 14145-50.
338. Yamamoto, H., Ihara, M., Matsuura, Y. & Kikuchi, A. (2003) Sumoylation is involved in beta-catenin-dependent activation of Tcf-4. *EMBO J* **22**, 2047-59.
339. Gomez-del Arco, P., Koipally, J. & Georgopoulos, K. (2005) Ikaros SUMOylation: switching out of repression. *Mol Cell Biol* **25**, 2688-97.
340. Lin, X., Liang, M., Liang, Y.Y., Brunicardi, F.C. & Feng, X.H. (2003) SUMO-1/Ubc9 promotes nuclear accumulation and metabolic stability of tumor suppressor Smad4. *J Biol Chem* **278**, 31043-8.
341. Steffan, J.S. et al. (2004) SUMO modification of Huntingtin and Huntington's disease pathology. *Science* **304**, 100-4.
342. Bailey, S.T. & Ghosh, S. (2005) 'PPAR'ting ways with inflammation. *Nat Immunol* **6**, 966-7.
343. Metivier, R., Penot, G., Hubner, M.R., Reid, G., Brand, H., Kos, M. & Gannon, F. (2003) Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* **115**, 751-63.
344. Pascual, G., Fong, A.L., Ogawa, S., Gamliel, A., Li, A.C., Perissi, V., Rose, D.W., Willson, T.M., Rosenfeld, M.G. & Glass, C.K. (2005) A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* **437**, 759-63.
345. Lin, D.Y., Fang, H.I., Ma, A.H., Huang, Y.S., Pu, Y.S., Jenster, G., Kung, H.J. & Shih, H.M. (2004) Negative modulation of androgen receptor transcriptional activity by Daxx. *Mol Cell Biol* **24**, 10529-41.
346. Lin, D.Y. et al. (2006) Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Mol Cell* **24**, 341-54.
347. Iniguez-Lluhi, J.A. & Pearce, D. (2000) A common motif within the negative regulatory regions of multiple factors inhibits their transcriptional synergy. *Mol Cell Biol* **20**, 6040-50.
348. Holmstrom, S., Van Antwerp, M.E. & Iniguez-Lluhi, J.A. (2003) Direct and distinguishable inhibitory roles for SUMO isoforms in the control of transcriptional synergy. *Proc Natl Acad Sci U S A* **100**, 15758-63.
349. Tian, S., Poukka, H., Palvimo, J.J. & Janne, O.A. (2002) Small ubiquitin-related modifier-1 (SUMO-1) modification of the glucocorticoid receptor. *Biochem J* **367**, 907-11.
350. Subramanian, L., Benson, M.D. & Iniguez-Lluhi, J.A. (2003) A synergy control motif within the attenuator domain of C/EBPalpha inhibits transcriptional synergy through its PIASy-enhanced modification by SUMO-1 or SUMO-3. *J Biol Chem*.
351. Ishov, A.M., Sotnikov, A.G., Negorev, D., Vladimirova, O.V., Neff, N., Kamitani, T., Yeh, E.T., Strauss, J.F.r. & Maul, G.G. (1999) PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. *J Cell Biol* **147**, 221-34.
352. Zhong, S., Muller, S., Ronchetti, S., Freemont, P.S., Dejean, A. & Pandolfi, P.P. (2000) Role of SUMO-1-modified PML in nuclear body formation. *Blood* **95**, 2748-52.

353. Best, J.L., Ganiatsas, S., Agarwal, S., Changou, A., Salomoni, P., Shirihai, O., Meluh, P.B., Pandolfi, P.P. & Zon, L.I. (2002) SUMO-1 protease-1 regulates gene transcription through PML. *Mol Cell* **10**, 843-55.
354. Shen, T.H., Lin, H.K., Scaglioni, P.P., Yung, T.M. & Pandolfi, P.P. (2006) The mechanisms of PML-nuclear body formation. *Mol Cell* **24**, 331-9.
355. Matunis, M.J., Zhang, X.D. & Ellis, N.A. (2006) SUMO: the glue that binds. *Dev Cell* **11**, 596-7.
356. Minty, A., Dumont, X., Kaghad, M. & Caput, D. (2000) Covalent Modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. *J Biol Chem* **275**, 36316-36323.
357. Hannich, J.T., Lewis, A., Kroetz, M.B., Li, S.J., Heide, H., Emili, A. & Hochstrasser, M. (2005) Defining the SUMO-modified proteome by multiple approaches in Saccharomyces cerevisiae. *J Biol Chem* **280**, 4102-10.
358. Wilson, V.G. & Rangasamy, D. (2001) Viral interaction with the host cell sumoylation system. *Virus Res* **81**, 17-27.
359. Orth, K., Xu, Z., Mudgett, M.B., Bao, Z.Q., Palmer, L.E., Bliska, J.B., Mangel, W.F., Staskawicz, B. & Dixon, J.E. (2000) Disruption of signaling by Yersinia effector YopJ, a ubiquitin-like protein protease. *Science* **290**, 1594-7.
360. Selkoe, D.J. (1999) Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* **399**, A23-31.
361. Li, Y., Wang, H., Wang, S., Quon, D., Liu, Y.W. & Cordell, B. (2003) Positive and negative regulation of APP amyloidogenesis by sumoylation. *Proc Natl Acad Sci U S A* **100**, 259-64.
362. Wang, L. & Banerjee, S. (2004) Differential PIAS3 expression in human malignancy. *Oncol Rep* **11**, 1319-24.
363. Cheng, J., Bawa, T., Lee, P., Gong, L. & Yeh, E.T. (2006) Role of desumoylation in the development of prostate cancer. *Neoplasia* **8**, 667-76.
364. Brown, D., Kogan, S., Lagasse, E., Weissman, I., Alcalay, M., Pelicci, P.G., Atwater, S. & Bishop, J.M. (1997) A PMLRARalpha transgene initiates murine acute promyelocytic leukemia. *Proc Natl Acad Sci U S A* **94**, 2551-6.
365. Muller, S., Miller, W.H.J. & Dejean, A. (1998) Trivalent antimonials induce degradation of the PML-RAR oncoprotein and reorganization of the promyelocytic leukemia nuclear bodies in acute promyelocytic leukemia NB4 cells. *Blood* **92**, 4308-16.
366. Shen, Z.X. et al. (2004) All-trans retinoic acid/As2O3 combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. *Proc Natl Acad Sci U S A* **101**, 5328-35.
367. Bowen, N.J., Fujita, N., Kajita, M. & Wade, P.A. (2004) Mi-2/NuRD: multiple complexes for many purposes. *Biochim Biophys Acta* **1677**, 52-7.
368. Imai, Y., Kimura, T., Murakami, A., Yajima, N., Sakamaki, K. & Yonehara, S. (1999) The CED-4-homologous protein FLASH is involved in Fas-mediated activation of caspase-8 during apoptosis. *Nature* **398**, 777-85.
369. Kittler, R. et al. (2004) An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. *Nature* **432**, 1036-40.
370. Woodage, T., Basrai, M.A., Baxevanis, A.D., Hieter, P. & Collins, F.S. (1997) Characterization of the CHD family of proteins. *Proc Natl Acad Sci U S A* **94**, 11472-7.
371. Barcaroli, D. et al. (2006) FLASH is an essential component of Cajal bodies. *Proc Natl Acad Sci U S A* **103**, 14802-7.
372. Milovic-Holm, K., Krieghoff, E., Jensen, K., Will, H. & Hofmann, T.G. (2007) FLASH links the CD95 signaling pathway to the cell nucleus and nuclear bodies. *EMBO J* **26**, 391-401.
373. Pugh, B.F. & Tjian, R. (1990) Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell* **61**, 1187-97.
374. Muller, C. & Leutz, A. (2001) Chromatin remodeling in development and differentiation. *Curr Opin Genet Dev* **11**, 167-74.
375. Schultz, D.C., Friedman, J.R. & Rauscher, F.J.r. (2001) Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. *Genes Dev* **15**, 428-43.
376. Xue, Y., Wong, J., Moreno, G.T., Young, M.K., Cote, J. & Wang, W. (1998) NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol Cell* **2**, 851-61.
377. Hong, W., Nakazawa, M., Chen, Y.Y., Kori, R., Vakoc, C.R., Rakowski, C. & Blobel, G.A. (2005) FOG-1 recruits the NuRD repressor complex to mediate transcriptional repression by GATA-1. *EMBO J* **24**, 2367-78.

378. Fujita, N., Jaye, D.L., Geigerman, C., Akyildiz, A., Mooney, M.R., Boss, J.M. & Wade, P.A. (2004) MTA3 and the Mi-2/NuRD Complex Regulate Cell Fate during B Lymphocyte Differentiation. *Cell* **119**, 75-86.
379. Le Guezennec, X., Vermeulen, M., Brinkman, A.B., Hoeijmakers, W.A., Cohen, A., Lasonder, E. & Stunnenberg, H.G. (2006) MBD2/NuRD and MBD3/NuRD, two distinct complexes with different biochemical and functional properties. *Mol Cell Biol* **26**, 843-51.
380. Brackertz, M., Gong, Z., Leers, J. & Renkawitz, R. (2006) p66alpha and p66beta of the Mi-2/NuRD complex mediate MBD2 and histone interaction. *Nucleic Acids Res* **34**, 397-406.
381. Williams, C.J., Naito, T., Arco, P.G., Seavitt, J.R., Cashman, S.M., De Souza, B., Qi, X., Keables, P., Von Andrian, U.H. & Georgopoulos, K. (2004) The chromatin remodeler Mi-2beta is required for CD4 expression and T cell development. *Immunity* **20**, 719-33.
382. Kasper, L.H., Boussouar, F., Ney, P.A., Jackson, C.W., Rehg, J., Van Deursen, J.M. & Brindle, P.K. (2002) A transcription-factor-binding surface of coactivator p300 is required for haematopoiesis. *Nature* **419**, 738-43.
383. Shi, X. et al. (2006) ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature* **442**, 96-9.
384. Bannister, A.J., Schneider, R., Myers, F.A., Thorne, A.W., Crane-Robinson, C. & Kouzarides, T. (2005) Spatial distribution of di- and tri-methyl lysine 36 of histone H3 at active genes. *J Biol Chem* **280**, 17732-6.
385. Ruthenburg, A.J., Li, H., Patel, D.J. & Allis, C.D. (2007) Multivalent engagement of chromatin modifications by linked binding modules. *Nat Rev Mol Cell Biol* **8**, 983-94.
386. Kirmizis, A., Santos-Rosa, H., Penkett, C.J., Singer, M.A., Vermeulen, M., Mann, M., Bahler, J., Green, R.D. & Kouzarides, T. (2007) Arginine methylation at histone H3R2 controls deposition of H3K4 trimethylation. *Nature* **449**, 928-32.
387. Lee, J.S. & Shilatifard, A. (2007) A site to remember: H3K36 methylation a mark for histone deacetylation. *Mutat Res* **618**, 130-4.
388. Shimono, Y., Murakami, H., Kawai, K., Wade, P.A., Shimokata, K. & Takahashi, M. (2003) Mi-2 beta associates with BRG1 and RET finger protein at the distinct regions with transcriptional activating and repressing abilities. *J Biol Chem* **278**, 51638-45.
389. Kino, T. & Chrousos, G.P. (2003) Tumor necrosis factor alpha receptor- and Fas-associated FLASH inhibit transcriptional activity of the glucocorticoid receptor by binding to and interfering with its interaction with p160 type nuclear receptor coactivators. *J Biol Chem* **278**, 3023-9.
390. Ness, S.A. (2003) Myb protein specificity: evidence of a context-specific transcription factor code. *Blood Cells Mol Dis* **31**, 192-200.
391. Chakalova, L., Debrand, E., Mitchell, J.A., Osborne, C.S. & Fraser, P. (2005) Replication and transcription: shaping the landscape of the genome. *Nat Rev Genet* **6**, 669-77.
392. Fraser, P. & Bickmore, W. (2007) Nuclear organization of the genome and the potential for gene regulation. *Nature* **447**, 413-7.
393. Sexton, T., Schober, H., Fraser, P. & Gasser, S.M. (2007) Gene regulation through nuclear organization. *Nat Struct Mol Biol* **14**, 1049-1055.
394. Ge, Q., Nilasena, D.S., O'Brien, C.A., Frank, M.B. & Targoff, I.N. (1995) Molecular analysis of a major antigenic region of the 240-kD protein of Mi-2 autoantigen. *J Clin Invest* **96**, 1730-7.
395. Seelig, H.P., Moosbrugger, I., Ehrfeld, H., Fink, T., Renz, M. & Genth, E. (1995) The major dermatomyositis-specific Mi-2 autoantigen is a presumed helicase involved in transcriptional activation. *Arthritis Rheum* **38**, 1389-99.
396. Tzavara, V., Stamoulis, K., Aroni, K.G., Kordossis, T. & Boki, K.A. (1997) Facial heliotrope rash as the initial manifestation of acute myelomonocytic leukemia. *Leuk Lymphoma* **25**, 393-8.
397. Nousari, H.C., Kimyai-Asadi, A., Huang, C.H. & Tausk, F.A. (2000) T-cell chronic lymphocytic leukemia mimicking dermatomyositis. *Int J Dermatol* **39**, 144-6.
398. Weismann, K. (2000) [Skin disorders as markers of internal disease. Paraneoplastic dermatoses]. *Ugeskr Laeger* **162**, 6834-9.
399. Carrera, C., Cibeira, M.T., Iranzo, P., Esteve, J., Nomdedeu, B. & Herrero, C. (2004) Dermatomyositis associated with acute myeloid leukaemia: a paraneoplastic association or a drug-induced phenomenon? *Acta Derm Venereol* **84**, 401-2.
400. Flotho, C., Coustan-Smith, E., Pei, D., Iwamoto, S., Song, G., Cheng, C., Pui, C.H., Downing, J.R. & Campana, D. (2006) Genes contributing to minimal residual disease in childhood acute lymphoblastic leukemia: prognostic significance of CASP8AP2. *Blood* **108**, 1050-7.
401. Barcaroli, D., Bongiorno-Borbone, L., Terrinoni, A., Hofmann, T.G., Rossi, M., Knight, R.A., Matera, A.G., Melino, G. & De Laurenzi, V. (2006) FLASH is required for histone transcription and S-phase progression. *Proc Natl Acad Sci U S A* **103**, 14808-12.

402. Jeong, E.G., Lee, S.H., Lee, H.W., Soung, Y.H., Yoo, N.J. & Lee, S.H. (2007) Immunohistochemical and mutational analysis of FLASH in gastric carcinomas. *Apmis* **115**, 900-5.
403. Park, J., Betel, D., Gryfe, R., Michalickova, K., Di Nicola, N., Gallinger, S., Hogue, C.W. & Redston, M. (2002) Mutation profiling of mismatch repair-deficient colorectal cncers using an in silico genome scan to identify coding microsatellites. *Cancer Res* **62**, 1284-8.
404. Geiss-Friedlander, R. & Melchior, F. (2007) Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol* **8**, 947-56.
405. Komatsu, T., Mizusaki, H., Mukai, T., Ogawa, H., Baba, D., Shirakawa, M., Hatakeyama, S., Nakayama, K.I., Yamamoto, H., Kikuchi, A. & Morohashi, K. (2004) Small ubiquitin-like modifier 1 (SUMO-1) modification of the synergy control motif of Ad4 binding protein/steroidogenic factor 1 (Ad4BP/SF-1) regulates synergistic transcription between Ad4BP/SF-1 and Sox9. *Mol Endocrinol* **18**, 2451-62.
406. Miller, A.J., Levy, C., Davis, I.J., Razin, E. & Fisher, D.E. (2005) Sumoylation of MITF and its related family members TFE3 and TFEB. *J Biol Chem* **280**, 146-55.
407. Berberich-Siebelt, F., Berberich, I., Andrulis, M., Santner-Nanan, B., Jha, M.K., Klein-Hessling, S., Schimpl, A. & Serfling, E. (2006) SUMOylation interferes with CCAAT/enhancer-binding protein beta-mediated c-myc repression, but not IL-4 activation in T cells. *J Immunol* **176**, 4843-51.
408. Lin, Y.S., Carey, M., Ptashne, M. & Green, M.R. (1990) How different eukaryotic transcriptional activators can cooperate promiscuously. *Nature* **345**, 359-61.
409. Carey, M., Lin, Y.S., Green, M.R. & Ptashne, M. (1990) A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives. *Nature* **345**, 361-4.
410. Chi, T., Lieberman, P., Ellwood, K. & Carey, M. (1995) A general mechanism for transcriptional synergy by eukaryotic activators. *Nature* **377**, 254-7.
411. Merika, M. & Orkin, S.H. (1995) Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Kruppel family proteins Sp1 and EKLF. *Mol Cell Biol* **15**, 2437-47.
412. Merika, M. & Thanos, D. (2001) Enhanceosomes. *Curr Opin Genet Dev* **11**, 205-8.
413. Guarente, L. (1995) Transcriptional coactivators in yeast and beyond. *Trends Biochem Sci* **20**, 517-21.
414. Chen, R.H. & Lipsick, J.S. (1993) Differential transcriptional activation by v-myb and c-myb in animal cells and Saccharomyces cerevisiae. *Mol Cell Biol* **13**, 4423-31.
415. Seneca, S., Punyammalee, B., Sureau, A., Perbal, B., Dvorak, M. & Crabeel, M. (1993) The carboxy-terminal domain of c-Myb activates reporter gene expression in yeast. *Oncogene* **8**, 2335-42.
416. Faus, H. & Haendler, B. (2006) Post-translational modifications of steroid receptors. *Biomed Pharmacother* **60**, 520-8.
417. Chen, W.Y., Lee, W.C., Hsu, N.C., Huang, F. & Chung, B.C. (2004) SUMO modification of repression domains modulates function of nuclear receptor 5A1 (steroidogenic factor-1). *J Biol Chem* **279**, 38730-5.
418. Pei, D.Q. & Shih, C.H. (1991) An "attenuator domain" is sandwiched by two distinct transactivation domains in the transcription factor C/EBP. *Mol Cell Biol* **11**, 1480-7.
419. Sakamuro, D. & Prendergast, G.C. (1999) New Myc-interacting proteins: a second Myc network emerges. *Oncogene* **18**, 2942-54.
420. Shen, H.C. & Coetzee, G.A. (2005) The androgen receptor: unlocking the secrets of its unique transactivation domain. *Vitam Horm* **71**, 301-19.
421. Chupreta, S., Holmstrom, S., Subramanian, L. & Iniguez-Lluhi, J.A. (2005) A small conserved surface in SUMO is the critical structural determinant of its transcriptional inhibitory properties. *Mol Cell Biol* **25**, 4272-82.
422. Baba, D., Maita, N., Jee, J.G., Uchimura, Y., Saitoh, H., Sugasawa, K., Hanaoka, F., Tochio, H., Hiroaki, H. & Shirakawa, M. (2005) Crystal structure of thymine DNA glycosylase conjugated to SUMO-1. *Nature* **435**, 979-82.
423. Vorbrueggen, G., Kalkbrenner, F., Guehmann, S. & Moelling, K. (1994) The carboxyterminus of human c-myb protein stimulates activated transcription in trans. *Nucleic Acids Res* **22**, 2466-75.
424. Bernardi, R. & Pandolfi, P.P. (2007) Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. *Nat Rev Mol Cell Biol* **8**, 1006-16.
425. Dellaire, G. & Bazett-Jones, D.P. (2004) PML nuclear bodies: dynamic sensors of DNA damage and cellular stress. *Bioessays* **26**, 963-77.
426. Lin, D.Y., Lai, M.Z., Ann, D.K. & Shih, H.M. (2003) Promyelocytic leukemia protein (PML) functions as a glucocorticoid receptor co-activator by sequestering Daxx to the PML oncogenic domains (PODs) to enhance its transactivation potential. *J Biol Chem* **278**, 15958-65.
427. de Ruijter, A.J., van Gennip, A.H., Caron, H.N., Kemp, S. & van Kuilenburg, A.B. (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J* **370**, 737-49.

428. Roscic, A., Moller, A., Calzado, M.A., Renner, F., Wimmer, V.C., Gresko, E., Ludi, K.S. & Schmitz, M.L. (2006) Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. *Mol Cell* **24**, 77-89.
429. Jones, P.L. & Shi, Y.B. (2003) N-CoR-HDAC corepressor complexes: roles in transcriptional regulation by nuclear hormone receptors. *Curr Top Microbiol Immunol* **274**, 237-68.
430. Gonda, T.J., Buckmaster, C. & Ramsay, R.G. (1989) Activation of c-myb by carboxy-terminal truncation: relationship to transformation of murine haemopoietic cells in vitro. *EMBO J* **8**, 1777-83.
431. Gonda, T.J., Ramsay, R.G. & Johnson, G.R. (1989) Murine myeloid cell lines derived by in vitro infection with recombinant c-myb retroviruses express myb from rearranged vector proviruses. *EMBO J* **8**, 1767-75.
432. Lane, T., Ibanez, C., Garcia, A., Graf, T. & Lipsick, J. (1990) Transformation by v-myb correlates with trans-activation of gene expression. *Mol Cell Biol* **10**, 2591-8.

Paper I

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Sæther T, Berge T, Ledsaak M, Matre V, Alm-Kristiansen AH, Dahle O, Aubry F, Gabrielsen OS. (2007) The chromatin remodeling factor Mi-2alpha acts as a novel co-activator for human c-Myb. *J Biol Chem* 282:13994-14005. doi:10.1074/jbc.M700755200

Paper II

II

Paper II has been removed from the thesis due to copyright restrictions. The published version can be downloaded here:

Alm-Kristiansen AH, Sæther T, Matre V, Gilfillan S, Dahle O, Gabrielsen OS. (2008) FLASH acts as a co-activator of the transcription factor c-Myb and localizes to active RNA polymerase II foci. *Oncogene* 27:4644-4656.
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Paper III

III

SUMO-conjugation of the transcription factor c-Myb controls cooperative behavior and induces an activator-to-repressor switch in the negative regulatory domain

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The transcription factor c-Myb plays a key role in controlling gene programs during proliferation and differentiation of hematopoietic cells. Several target genes are activated by c-Myb in synergy with other transcription factors. Since the synergy properties of some factors are restricted by sumoylation (synergy control) and c-Myb is being sumoylated, we investigated these characteristics of c-Myb. By using a set of dedicated reporters we quantified the synergy behavior of c-Myb and found the factor to be subject to strong synergy control linked to SUMO-conjugation in its negative regulatory domain (NRD). When the ability of c-Myb to become sumoylated was modulated in various ways, its synergy behavior changed accordingly. Interestingly, AMV v-Myb appears to have escaped synergy control and a rather high synergy factor was measured for this oncogenic version of c-Myb. The search for a mechanism revealed a SUMO-controlled switch in the function of NRD. When NRD is sumoylated, it acts negatively by reducing the activity of c-Myb. When SUMO is removed, NRD switches into a transactivation domain (TAD), providing the factor with a second TAD. We propose a new model for c-Myb having two TADs, one centrally located and constitutively active and one in NRD being under SUMO-control. We also propose a new model for SUMO-mediated synergy control in which SUMO restrains the number of active TADs and thus the available co-activator interaction surfaces. In this model, SUMO controls synergy by determining the number and strength of independent and active TADs associated with a promoter through the bound transcription factors.

Synergy between transcription factors operating together on complex promoters is a well-known phenomenon. Several models have been proposed to explain this more-than-additive activity of multiple activators. Key concepts are multiplicity of contacts to the basal transcription apparatus (1,2) promoting assembly of the pre-initiation complex (PIC) (3), physical interactions between transcription factors (4) particularly emphasized in the enhanceosome model (5), co-activators harboring distinct domains that interact simultaneously with different factors (6), and cooperative interactions of transcription factors with nucleosomal DNA (7). Activators able to stimulate distinct steps in the transcription process, such as initiation and elongation, may also contribute to concerted action and synergy (8). In general terms, synergy appears to be intrinsic to the transcription process, being related to the multiplicity of interactions necessary to assemble an active PIC at the transcription start site (TSS) and unleash the productive elongation by RNA polymerase II. This makes synergy an ideal target for control of transcriptional output.

An interesting twist to the phenomenon of synergy was the finding of a specific negative control mechanism. Iniguez-Lluhi and Pearce identified a short protein motif in the glucocorticoid receptor (GR) that mediated "Synergy control" (SC), by acting as a disruptor of synergy on promoters with multiple response elements, but not so on single response elements (9). Mutations of this motif unleashed a strong synergistic behavior of GR at compound, but not at single, response elements. It soon became apparent that the SC motif was indeed a SUMO-conjugation site and that the disruption of synergy was caused by sumoylation of the factor at that site (10,11).

This role of SUMO (Small ubiquitin-related modifier) as a disruptor of synergy has been extended to other transcription factors such as Ets and C/EBPα (9,12). Still, compared to the rapidly expanding literature on SUMO only a tiny fraction of papers have addressed its synergy-controlling properties.

SUMO family proteins function by becoming covalently linked to a variety of proteins, including many nuclear regulators of key processes such as transcription, nuclear transport, chromatin structure, and DNA repair (13-16). The modification by SUMO is a highly dynamic process, controlled by the balance between a set of conjugation enzymes, analogous to those of the ubiquitin pathway, and a set of SUMO-specific proteases.

c-Myb is a key transcription factor controlling proliferation and differentiation of early hematopoietic progenitor cells, as well as being a regulator of similar processes in other tissues such as colon (17,18). c-Myb becomes sumoylated at two sites within its negative regulatory domain leading to a severe drop in its activity (19,20). Interestingly, both SUMO-conjugation sites are deleted in the oncogenic variant AMV v-Myb (19). The molecular mechanism by which SUMO is controlling c-Myb activity is poorly understood. Interestingly, synergy is a well-documented aspect of c-Myb action. The factor has been reported to activate promoters in synergy with several other transcription factors such as Ets, C/EBP, PU.1, Pax-5 and CBF (core binding factor) (21-28). Consistently, many of the genes activated by c-Myb appear to be controlled by compound promoters harboring multiple recognition sites both for c-Myb and for other cooperating factors.

Given the role of SUMO as a disruptor of synergy for other transcription factors, we reasoned that studying its role in synergy control of c-Myb might lead to a better understanding of the mechanisms by which SUMO controls c-Myb action. In this work we show that c-Myb is subject to a strong synergy control, tightly linked to its level of SUMO-conjugation, and that this synergy control is abolished in AMV v-Myb. This control is expected to be promoter-specific related to the multiplicity of response elements and complexity of the activated promoters. Accordingly, we show that chromatin embedded target genes of c-Myb respond differentially to SUMO-control in hematopoietic cells. Furthermore, we show that SUMO affects the transactivation properties of the factor by switching off an activation function in its negative regulatory domain, contributing to the amplitude of activation. We propose a dual role for sumoylation of c-Myb in controlling both the transactivation properties and the synergy behavior of c-Myb. This leads to a considerable dynamic control of c-Myb activity.

EXPERIMENTAL PROCEDURES

Reporter plasmids. pGL4-1×MRE(GG)-MYC, pGL4-2×MRE(GG)-MYC, pGL4-3×MRE(GG)-MYC, pGL4-4×MRE(GG)-MYC, pGL4-5×MRE(GG)-MYC were constructed in two steps. Oligos, containing a sequence from human *MYC* P2 core promoter, were first inserted into the *Kpn*I/*Sma*I site of pGL4 basic (a designed derivative of pGL3 Basic (E1751; Promega)) where the MCS was changed by oligo-insertion to contain sites for *Mlu*I, *Xho*I, *Kpn*I, *Sma*I and *Bgl*II). Then oligos were designed to contain one, two, three, four or five Myb-responsive elements (MREs TAACGG) with a spacing of four bp (TTTT) giving a phasing of ten bp between the MREs (i.e. MREs starting in position +1, +11, +21 etc). Duplex oligos were subcloned into *Mlu*I/*Xho*I site of the plasmid. Oligo insertions were verified by sequencing. In the same way, pGL4-4×MRE(GG)abab-MYC was constructed by inserting an oligo containing four MREs in which the phasing was 15 bp between the elements. The reporter pGL4-3×MRE(GG)aab-MYC contains three MREs in which the phasing is 10 bp between the first two and 15 bp between the second and third MRE.

The *E1b* driven Gal4p responsive luciferase reporter, pG5E1bLuc, containing five binding sites for the yeast transcription factor Gal4p upstream of an Adenovirus E1b TATA-box and a luciferase gene, used in the Gal4 tethering assays is described (29). The pGL3b-5GRE-SNRPN is an *SNRPN*-driven Gal4p-responsive luciferase reporter (30).

Mammalian expression plasmids. The mammalian expression vectors pCIneo-hcM-HA and pCIneo-hcM-HA-2KR (encoding wild type and sumoylation deficient c-Myb, respectively), pCIneo-hcM-HA-K503R and pCIneo-hcM-HA-K527R (single sumoylation sites mutated) have been described (19). c-Myb

mutants pCIneoB-hcM-HA-E505A, pCIneoB-hcM-HA-E529A and pCIneoB-hcM-HA-E505/529R (abbreviated 2EA) were generated using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) on a subfragment of human *MYB*, before subcloned into pCIneoB-hcM-HA. pCIneo-hcM-2KR-SUMO-1 was constructed by PCR amplification and modification of SUMO-1 from the appropriate IMAGE clone, followed by subcloning in-frame into pCIneo-hcM-HA-2KR (between *Psh*AI and *Sal*I), loosing the HA tag, but gaining SUMO-1 in the expressed product. pCIneo-hcM-VP16 was made by PCR amplification of the herpes simplex virus VP16 transactivation domain from pDBD11 (31). The VP16-TAD region was subcloned in-frame into pCIneo-hcM. pCIneo-hcM-VP16–2KR has been described (30).

pCIneo-AMV encoding the (AMV v-Myb protein, residue 72–440 in chicken c-Myb) is described in (32), while the pCIneo-hcM-ΔNRD-HA has been described elsewhere (Alm-Kristiansen *et al*., *submitted* 2007).

The c-Myb expression constructs with the central TAD deleted (pCIneoB-hcM-ΔT1-HA and pCIneoB-hcM-ΔT1-HA-2KR) were made from a cDNA in Bluescript lacking an internal fragment (*Hpa*I to *Sma*I) encoding human c-Myb amino acids 229-325. The *Eco*RI-*Bgl*II fragment from pBS-ΔSE-hcM-ΔT1 was subcloned between the corresponding sites in pCIneoB-hcM-HA and pCIneoB-hcM-HA-2KR, respectively.

The mammalian expression vectors for Gal4p-DBD fused to HA-tagged human c-Myb NRD (pCIneoB-GBD2-NRD-HA; coding for amino acid residue 410 to 640) were made by PCR amplification of the corresponding sequence in pCIneo-hcM-HA and subcloning of this fragment into pCIneoB-GBD2 (described earlier (30)) between *Sal*I and *Not*I. pCIneoB-GBD2-NRD-HA-2KR and -2EA were made by subcloning the *Bgl*II-*Not*I fragment from pCIneo-hcM-HA-2KR and -2EA, respectively, into pCIneoB-GBD2-NRD-HA. The Gal4p-DBD NRD-SUMO fusion proteins were made by the same strategy using pCIneo-hcM-2KR-SUMO-1 and pCIneo-hcM-SUMO-1 (30).

The SENP1 expression plasmids pFlag-CMV-SENP1 and pFlag-CMV-SENP1-mutant (R630L, K631M) were a kind gift from Dr. E.T. Yeh (33).

Cell Culture, Transfection, and Luciferase Assays. CV-1 and COS-1 cells were grown as described (34). HD11 cells were grown in Iscove's Modified Dulbecco's Medium supplemented with 8 % heat-inactivated fetal bovine serum, 2 % heat-inactivated chicken serum and antibiotics, at 37 °C and 5 % CO_2. All three cell lines were transiently transfected with the indicated plasmids (0.2 µg when nothing else is mentioned) using FuGENE6 (RocheApplied Science) 24 h after seeding. For the luciferase assays, CV-1 cells were harvested 24 h after transfection and lysed in Passive Lysis Buffer (Promega). Luciferase assays were performed in triplicate (24 well trays; 2×10^4 cells/well) using Luciferase Assay Reagent (Promega), and data from at least three independent transfection experiments are presented.

For the Trichostatin A (TSA) treatment of CV-1 cells TSA (Sigma) was dissolved in EtOH and added to the culture medium, 8 h after transfection, at a final concentration of 100 nM. After 14 h of incubation with TSA the cells were harvested and luciferase assays performed.

Expression of Myb proteins for EMSA. COS-1 cells were transfected as described above with 5 µg DNA (pCIneo-hcM-HA, pCIneo-hcM-2KR-HA or pCIneo-hcM-2KR-SUMO-1) per 100-mm plates, seeded with 1×10^6 cells the day before. 24h after transfection the cells were washed twice in 1×PBS on ice before lysis in 500 µl modified Buffer F (10 mM Tris-HCl [pH 7.05], 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 mM $ZnCl_2$, 100 mM Na_3VO_4, 1 % Triton X-100, 1 mM PMSF and 1 mM DTT) supplemented with Complete Protease Inhibitor™ (Roche Applied Science) (35). The lysates were then centrifugated for 30 min at 4 °C, before aliquots of the supernatant were frozen in liquid N_2 and stored at -80 °C.

Electrophoretic Mobility Shift Assay. DNA binding was monitored by EMSA as previously described (36). The oligonucleotide used for Myb binding is based on the MRE A site in the *mim-1* promoter (37); 5'-GCATTATAACGG-TTTTTTAGCGC-3. The probe was labeled with γ-^{32}P-ATP and purified as described in (38). COS-1 cell lysates (1-9 µl) with the different Myb proteins were adjusted to equal volumes with Buffer F before incubation in CG-buffer (20 mM Tris-HCl [pH 8.0], 0.1 mM

EDTA, 10 % glycerol), with final NaCl concentration 75 mM and 1 µg poly[dI-dC]. 5'-γ-^{32}P-labeled MRE oligonucleotide probe (20 fmol) were added and the binding mixture (total volume 20 µl) was incubated for 10 min at room temperature before electrophoresis. Binding reactions were run on 6 % 0.5×TBE, 5 % glycerol PA-gels at 4 °C.

Western blotting and antibodies
Cell lysates from COS-1-transfected cells were run on a 10 % SDS PA-gel and subjected to Western blotting using Hybond P membranes (GE Healthcare) and ECL plus kit (GE Healthcare) for detection. The murine Myb 5e11 antibody (39) was used as primary antibody and anti-mouse IgG-HRP (NA931, GE Healthcare) was used as secondary antibody.

RNA isolation and quantitative RT-PCR.
HD11 cells were transfected as described above with 2 µg DNA (pCIneo-hcM-HA, pCIneo-hcM-2KR-HA or empty pCIneo vector) in 6-well trays (seeded with 5×10^5 cells per well the day before). 24h after transfection the cells were harvested and total RNA extracted with Trizol reagent (Invitrogen) and subsequent DNase treatment and purification of the RNA using RNeasy columns (Qiagen). 3µg of RNA for each sample were used for reverse transcription using the Superscript™ III system (Invitrogen). The cDNA obtained was subjected to real-time PCR analysis to determine the expression of three c-Myb target genes: *mim*-1, *lysozyme* and *Pdcd4*, using the LightCycler DNA MasterPlus SYBR Green Kit (Roche). A standard curve made from serial dilutions of cDNA was used to calculate the relative amount of these mRNAs in each sample. These values were normalized to the relative amount of the reference gene *HPRT* in the same samples, calculated from a standard curve established in the same way. The cellular transfections were performed in triplicate and the experiment was repeated three times. Primer sequences are available upon request.

RESULTS

Synergy behavior of c-Myb and its relation to SUMO-conjugation.

Given that c-Myb is being sumoylated and that its activity is significantly enhanced by removal of SUMO-conjugation sites (19,20), we asked whether c-Myb was subject to the phenomenon of synergy control. Systematic analysis of this type of behavior requires a dedicated set of reporter constructs with defined changes in the multiplicity of response elements. Hence, we constructed a set of reporter plasmids based on the pGL3 backbone with identical core promoters (from *MYC* P2), activated by one to five copies of an optimal MRE (Myb response elements) each with a phasing of ten bp (Fig. 1A). In a systematic EMSA study of recombinant c-Myb binding to differently spaced MREs, we had observed that a phasing of six bp caused interference from one bound factor on the binding of a second, while a phasing of ten bp did not (data not shown). When reporters with one to five MREs were compared in effector-reporter assays in transfected CV-1 cells, wild type c-Myb activated the various reporters only with minor differences (Fig. 1B). In contrast, the SUMO-conjugation negative mutant, c-Myb 2KR, showed a remarkable increase in activity when the number of MREs were augmented (Fig. 1B). In order to be able to quantify this difference in synergistic behavior, we defined a relative "synergy factor" (SF) as the ratio of reporter activity with four MREs to the activity with one MRE, divided by four (the latter to obtain ratios independent on the number of MREs). A simple proportional increase in reporter activity with the number of MRE should then give an SF = 1, while a positive synergy will result in SF > 1. An SF < 1 would then imply a less than additive effect, which might be termed "negative synergy" (illustrated in Fig. 1C). We decided to calculate the SFs based on four MREs, but very similar SFs were obtained when we calculated ratios based on three or more MREs (data not shown). Using this simple quantification scheme, we compared wild type c-Myb with mutants in one or both SUMO-conjugation sites (Fig. 1D). Wild-type c-Myb displayed in fact a negative synergy (SF = 0.26) while the SUMO-negative c-Myb 2KR showed a strong positive synergy (SF = 3.9). Single mutants, retaining one of the two SUMO-conjugation sites, showed intermediate levels (K503R: SF = 0.98; K527R: SF = 0.72). These results suggest that the synergistic behavior of c-Myb is highly dependent on its ability to become sumoylated.

To investigate this association further, we asked whether the strong synergy of c-Myb 2KR could be reduced when SUMO-1 is

covalently fused to the C-terminal of c-Myb 2KR, mimicking the sumoylation of the protein. Figure 1E shows that this is indeed the case. The c-Myb-2KR-SUMO-1 fusion gave a strong reduction in synergy (SF = 0.95) when compared to the non-fused 2KR protein (SF = 3.92). The SF value of the fusion is in fact very similar to the SF of the single mutants (K503R and K527R; Fig. 1D) also harboring one SUMO moiety in their conjugated state. This observation strongly supports a role for SUMO in controlling the synergy behavior of c-Myb. Consistent with this, ectopic expression of SENP1 (a SUMO-protease) enhanced the synergy behavior of wild type c-Myb (SF increased from 0.26 to 2.8) while a protease-dead SENP1 had no effect (Fig. 2A). As expected, the synergy behavior of c-Myb 2KR, lacking functional conjugation sites, was not influenced by SENP1. We also compared alternative mutants (ΨKXE → ΨKXA) to abolish SUMO-conjugation, while allowing other Lys-directed modifications to occur. The 2EA mutant (E505A+E529A) severely reduced sumoylation of c-Myb (data not shown) and caused a strong increase in synergy (SF = 2.46; Fig. 2B). Taken together, these data suggest that SUMO-conjugation is the major determinant of synergy behavior of c-Myb as revealed by this MRE-multiplicity assay.

The oncogenic activated AMV v-Myb harbors both point mutations and N- and C-terminal deletions, removing both SUMO-conjugation sites. We expected the latter deletion to affect the synergy behavior of v-Myb. The cumulative effects of the other mutations however were less predictable in a synergy assay. As shown in Fig. 3A, v-Myb displayed in fact the strongest synergy of all Myb-variants tested so far (SF = 4.78) suggesting that escaping synergy control might be an important strategy during oncogenic activation.

There have been reports on viral strategies to overcome the effect of the SUMO-conjugation system (40). Since VP16 is a viral protein that in model systems often is fused to transcription factors to transform them into strong activators (41), we tested the synergy behavior of a c-Myb-VP16 fusion. As shown in Fig. 3B the fusion of VP16 to c-Myb clearly overrides the negative synergy, increasing the SF of c-Myb from 0.26 to 1.39. This ability of VP16 to confer positive synergy behavior on its target factor may relate to the increased number of TADs provided (see below).

In the reporters used to calculate SF values, the phasing of the MREs was 10 bp, causing the bound c-Myb activators to have the same helical orientation on the promoter. To see whether the helical orientation is of any importance for synergy, we designed a 4×MRE reporter with a phasing of 15 bp (pGL4-4×MRE(GG)abab-MYC) to give an alternating helical orientation (Fig. 3C). While the SF values obtained with the SUMO-negative c-Myb 2KR did not change with helical orientation, the lower values obtained with wild type c-Myb did in fact change. The level of "negative synergy" became more relaxed when the helical orientation became alternating (SF = 0.83) compared to when all elements were oriented on the same side of the DNA helix (SF = 0.26). This may reflect a different mechanism responsible for cooperation generating positive synergy as compared to the mechanism restricting synergy.

Relevance of SC to resident promoters

The Myb-responsive model promoters used for assaying the SUMO-dependence of synergy were controlled by one single transcriptional activator. Most natural promoters on the other hand are more complex and often activated by a combination of transcription factors, several of which may be SUMO-conjugated. The activation of a natural promoter through desumoylation would be expected to involve removal of SUMO from various transcription factors. Unfortunately, this is not easy to mimic in an experimental setup. However, even though Myb-regulated promoters also are under the control of other factors, we tried to address the relevance of the SUMO-mediated synergy control of c-Myb in a more physiological context by comparing the activation of endogenous target genes by wild type c-Myb and c-Myb 2KR. For this purpose we used c-Myb negative chicken macrophage HD11 cells since these cells express C/EBPβ known to cooperate with c-Myb in transcriptional activation (24,42). Activation of three different genes reported to be targets of c-Myb was monitored by real-time PCR. As shown in Figure 4A, co-transfection of c-Myb wild type or 2KR mutant resulted in a differential activation of *mim*-1, as expected if several MREs contribute to the activity of the

chromatin embedded promoter. For the *lysozyme* gene (Fig. 4B) the difference between c-Myb wild-type and 2KR was less pronounced, suggesting that fewer MREs are involved. Only low levels of activation and no obvious difference between 2KR and wild-type, was observed with the *Pdcd4* gene (Fig. 4C). However, in this last case, the low level of *Pdcd4* activation made it difficult to assess the differential activation precisely. In addition to the number of MREs, these promoters may differ is the contribution to activity from c-Myb versus from other factors. This may explain why we observe the strongest synergy effect in the promoter showing the strongest activation by c-Myb. We conclude that natural promoters differ significantly in their synergy behavior when c-Myb is compared in two states. We expect however that a larger difference would have been observed if we had been able to compare two or more cooperating factors in both SUMO-conjugated and non-conjugated states.

In search for a mechanism

Being confident that the synergy property of c-Myb is controlled by SUMO, we asked what mechanism might be involved. We reasoned that at least three possibilities should be explored. First, SUMO-conjugation of c-Myb may alter its intrinsic properties, converting c-Myb into a less potent factor with reduced ability to synergize with other factors. If so, this might be caused either by reduced specific DNA-binding or by impaired transactivation properties. In addition to these two mainly intrinsic mechanisms, SUMO-conjugation may, by adding a new interaction surface to c-Myb, lead to the recruitment of a repressor causing the transactivation output to be impaired.

This last possibility was addressed by taking into consideration reports showing that SUMO-conjugation in other systems lead to recruitment of HDAC6 (43) or HDAC-2 (44). If sumoylation of c-Myb leads to recruitment of HDACs and consequently a decrease transcription, then the inhibition of HDACs by TSA treatment should result in increase of the SF. We therefore measured the synergy behavior of c-Myb in the presence and absence of TSA. We observed, however, no significant alteration in the SF for c-Myb wild-type after addition of TSA (Fig. 5), suggesting that HDACs probably do not account for the phenomenon. We measured the activity of the *SNRPN* promoter under the same conditions to assure the efficiency of the TSA treatment. As shown in Fig 5B, this promoter became significantly activated by TSA, making us confident that the TSA treatment did work. We cannot from the TSA-insensitivity of the synergy behavior exclude a repressor-recruitment model if the associated protein causes repression by an HDAC-independent mechanism. Nevertheless, we chose to proceed by exploring the two intrinsic mechanistic possibilities, modified transactivation or altered DNA-binding.

To monitor DNA-binding properties, we expressed in COS-1 cells the three Myb variants used above to calculate SFs: c-Myb wild type, c-Myb 2KR, and c-Myb-2KR-SUMO1. We evaluated the amount of protein by Western analysis (Fig. 6 lower panel) and performed EMSA. With equal input of c-Myb proteins, the specific DNA-binding observed was similar for c-Myb wild type and 2KR, as well as for the SUMO-fusion protein (Fig. 6 upper panel), suggesting that the SUMO moiety fused to the C-terminal of c-Myb had no significant influence on the activity of the DNA-binding domain localized in the N-terminal of the protein.

We then proceeded to study if the intrinsic transactivation properties of c-Myb were modulated by sumoylation. SUMO may by itself act as a kind of general repressive domain as suggested by its behavior in Gal-tethering assays (44,45) and through its reported role in HDAC recruitment (43,44). We asked whether SUMO conjugated to c-Myb might have a more specific effect in controlling the TAD functions of the factor. First, we addressed whether the region becoming sumoylated in c-Myb by itself might contribute to the transactivation properties of c-Myb. A previous report showed that this region when tested in yeast in fact displays TAD activity (46).

The classical way of monitoring TAD-function is by fusing the domain under investigation to Gal4p and monitoring the induced activation of a Gal4p-responsive reporter in transfected cells. When we tested the NRD region of c-Myb (amino acids 410-640) in such a system, we saw that the wild type version of NRD did not activate, but rather had a weak repressive effect (Fig 7A). However, when the SUMO-conjugation sites were mutated (2KR) in the same domain, we

observed that the NRD was no longer acting negatively in this assay, but had switched into a positive transactivation domain (Fig 7A). To make sure that this remarkable observation was not some peculiarity related to the minimal E1B promoter in the pG5E1bLuc reporter used, we repeated the experiment with an SNRPN-driven Gal4p-responsive luc reporter, observing essentially the same effect (data not shown). To confirm that the observed switch was indeed related to the elimination of SUMO-conjugation, we tested out the E-A mutants instead of the K-R mutants in the same system. Also in this case, we observed the same change in the properties of the NRD becoming an activating domain upon reduced sumoylation (Fig 7A). The 2EA mutant always gave somewhat weaker response than 2KR, probably because the conjugation sites remain intact and the reduction in sumoylation is not totally abolished (data not shown). The fusion of SUMO-1 to the active 2KR-version of the NRD eliminated its TAD function totally, again supporting that the switch behavior is closely related to SUMO-conjugation. Finally, we analyzed the Gal-NRD fusion (wild-type) in the presence of overexpression of SENP1. As shown in Fig 7B, this SUMO-protease was able to induce the switch of the NRD from a weak repressor to a positive activator. While the wild type NRD changed 50-fold in activity upon overexpression of SENP1, the already derepressed 2KR version did not increase more than the background change. It should be noticed that all the Gal-responsive reporters used contain arrays of Gal-responsive elements (5×GRE with 10 bp phasing), making the switch observed relevant for the phenomenon of synergy control.

Having found that c-Myb NRD behaved as a SUMO-repressed TAD when studied separately, we asked if this property also contributed to the overall transactivation potential of the full-length protein. The implicated model is that c-Myb has two TADs, one constitutive centrally located in the protein, and a second one SUMO-repressed in the NRD region, functioning as an internal repressor in the wild type protein (illustrated in Fig. 8). To test this model, we first compared the level of activity of three c-Myb constructs with different combinations of these two TADs. Consistent with earlier reports, the c-Myb-ΔNRD was much more active than the full-length protein (Fig. 8). This activation by deletion shows the existence of a negative influence from the NRD region and is in fact the main reason for the "NRD" designation (47,48). Interestingly, when c-Myb 2KR was measured in parallel, we clearly saw that the elimination of SUMO-conjugation creates an even more active protein than c-Myb ΔNRD, as expected if the silenced second TAD is switched on in this construct. Thus, the c-Myb 2KR has not only lost its internal repressor function but behaves as a protein with two TADs (Fig. 8).

To test this model further, we reasoned that if the NRD contains a repressed transactivation domain that becomes active upon removal of SUMO-conjugation, we should be able to rescue a defect introduced in c-Myb caused by deletion of its constitutive central transactivation domain, simply by reactivating its silenced TAD in NRD. In Figure 9 we have compared c-Myb wild type, c-Myb with its central TAD deleted, and c-Myb with the TAD-deletion in combination with the 2KR mutation. Clearly, the expected rescue is observed showing that also in the full-length protein the NRD can be activated into a second TAD which will contribute to the overall transactivation potential of the c-Myb protein.

Synergy and number of TADs

Given that SUMO-conjugation leads to suppression of a second TAD-function in c-Myb, it appears quite probable that derepression of this TAD upon removal of SUMO, makes an important contribution to the enhanced synergy of c-Myb 2KR. An obvious model, combining the synergy properties of the c-Myb variants and the switch-property of NRD, would be that the efficiency of synergy as measured by the synergy factor, relates to the number and strength of independent and active TADs brought to a promoter through the bound transcription factors. If this is correct, we should be able to measure increasingly larger SFs for c-Myb variants harboring increasing number (and strengths) of independent and active TADs. To test this hypothesis we measured synergy factors for two additional c-Myb variants: c-Myb ΔNRD (harboring one TAD) and c-Myb-2KR-VP16 (harboring three TADs). These were compared with wild type c-Myb (harboring one partially repressed TAD) and c-Myb 2KR (harboring two TADs). As shown in Fig. 10, we observed

the expected SF increase when number and strengths of TADs increased. The deletion c-Myb ΔNRD with one TAD relieved from the repressive influence of NRD gave a higher SF than wild type, but still well below that of c-Myb 2KR harboring two TADs. The c-Myb-2KR-VP16 fusion protein, harboring three TADs, showed an extremely large SF of 35. This variant then was 140-fold more active on a promoter with four MREs than on a promoter with a single MRE.

We conclude that removal of SUMO-conjugation in c-Myb unleashes the potential of c-Myb to synergize on compound promoters. One important contribution to this increased synergy appears to be the derepression of a silenced transactivation domain in the NRD of c-Myb becoming active upon removal of SUMO-conjugation. We cannot exclude that additional mechanism also are involved, but the NRD switch certainly makes an important contribution to the large differences observed between wild type and SUMO-negative c-Myb on complex promoters.

DISCUSSION

Synergy is an inherent property of the transcription system. In contrast to the ribosome, no large preassembled structure takes care of mRNA synthesis. Rather a multitude of factors act together in a dynamic interplay to initiate transcription. The phenomenon of synergy appears to be a natural consequence of this design, being related to the multiplicity of interactions necessary to assemble an active pre-initiation complex at the TSS and unleash the productive elongation by RNA polymerase II. Since the initiation of transcription is the outcome of multiple weak interactions, any slight modulations of these may cause a large change in output. Thus, the phenomenon of synergy appears to be an ideal target for transcriptional control. We have in this work shown that c-Myb appears to be a rather weak activator, but with inherent potential to become a quite strong one. This property is revealed by two phenomena studied in this work. First we have shown that removal of SUMO-conjugation in c-Myb unleashes the potential of c-Myb to synergize on compound promoters. The larger the number of Myb-responsive elements, the larger the difference in Myb-induced reporter activation between wild-type c-Myb and its SUMO-conjugation negative mutant. All experimental modulations that changed the level of SUMO-conjugation led to the expected changes in synergy behavior, firmly linking the ability of c-Myb to synergize with its ability to become sumoylated. Having established this link, we further show that a major contribution to the increased synergy caused by SUMO-removal is the derepression of a silenced transactivation domain in the negative regulatory domain of c-Myb. This domain is able to switch from acting as an internal repressive domain into an active transactivation domain upon removal of SUMO-conjugation. Our data suggest a new model for c-Myb in which the factor has two TADs, the established central TAD acting in a constitutive fashion, and a second inducible TAD being repressed by SUMO-conjugation. The latter provide c-Myb with an inherent potential to change from a rather weak activator into a potent transactivating factor harboring a double set of TADs. This model also implies that sumoylation is an important regulatory mechanism of the activity of c-Myb.

The dual TAD data shed light on an intriguing observation reported some time ago showing that the NRD region of c-Myb causes activation in yeast but repression in animal cells (46). This difference may now rather be linked to differences in the SUMO apparatus between the two biological systems, either with respect to SUMO-conjugation or to presence of SUMO-binding repressors. Because of this the SUMO-mediated NRD-repression mechanism probably does not function in yeast.

The link between the ability of a transcription factor to synergize on a complex promoters and it being relieved from SUMO-conjugation, was first observed in studies of the glucocorticoid receptor (GR)(9,10), and later extended to other factors such as C/EBPα, SF-1 and MITF (12,49,50). We show in this work that c-Myb may be added to the growing list of synergy-controlled transcription factors. Concerning mechanisms, several possibilities, such as SUMO-mediated recruitment of co-repressors, have been proposed (9,10), but none of the previous studies have demonstrated a specific mechanism explaining the phenomenon of synergy control. We have made several observations that have implications for how a mechanistic model may look like.

A mechanistic model has to take into account that the phenomenon of synergy is related to the multiplicity of interactions during

activation of a promoter. Therefore, SUMO-mediated restriction of synergy would be expected to be caused either by a SUMO-induced blocking of productive interactions or a SUMO-induced generation of repressive interactions. The first would be the case if SUMO-conjugation directly obstructed the association with a co-activator (illustrated in Fig. 11A) or indirectly induced a conformational change reducing the affinity for a co-activator. The latter would be the case if SUMO-conjugation of a transcription factor created affinity for a co-repressor (Fig. 11B). We reason that the synergy behavior observed, points to *multiplicity* as an important aspect that has to be incorporated into the mechanistic model. One way of incorporating this is to assume the recruitment of multivalent co-repressor able to interact with arrays of SUMO-conjugated factors. Multivalent recruitment of co-activators have for some time been the classical model for explaining synergistic activation of promoters (Fig. 11C). In an obstruction model, such multivalent co-activator recruitment would be very efficiently killed if all the recruiting factors were modified by a blocking SUMO-peptide (Fig. 11A). In line with this, recruiting a SUMO-protease would be a way to switch on a promoter kept silent through sumoylation of bound transcription factors.

Our finding that the NRD region of c-Myb has the particular ability to switch from acting negatively to acting positively depending on sumoylation status is a novel element that may clarify the mechanism. Based on these switch-properties, we propose a more specific model for the synergy control phenomenon operating on c-Myb. In this model synergy is determined by the number and strength of independent and active TADs associated with a promoter through the bound transcription factors. Under conditions of normal sumoylation, c-Myb contains one TAD partially repressed by NRD and the level of synergy is rather low (SF = 0.26). Upon reduced sumoylation, as mimicked in the 2KR mutant or by ectopic expression of SENP1, the second TAD is turned on and the central TAD is relieved of its repression. Now, c-Myb will operate with two active TADs and therefore show higher synergy (SF = 3.9 and 2.8 respectively). A similar positive synergy is seen in the c-Myb-VP16 fusion protein with two TADs, one weak and one very strong (SF = 1.4). A factor with three independent TADs, as seen with c-Myb 2KR VP16 displays an extremely high synergy (SF = 35). In this model the number of TADs is a critical determinant of synergy. The activation potential of a single TAD may be enhanced by mutation, as seen in the case of the ANAA mutant in c-Myb (Sæther *et al*., in preparation). However, only a modest increase in synergy factor is observed with this mutant, probably because the number of TADs remains unaltered in the mutant protein.

A rationale for linking synergy to the number of TADs is that these domains most likely act as individual interaction domains. Therefore, two interaction domains would logically lead to more efficient co-activator recruitment than a single one, three even more so etc. A promoter-centered view is appropriate here. When two or three transcription factors cooperate, this means that they provide a promoter with two or three TADs resulting in a gradually increased efficiency of co-activator recruitment (Fig. 11C). A single factor presenting one or two TADs would in a similar fashion provide the promoter with one or two TADs each contributing to an increased efficiency of co-activator recruitment (Fig. 11D and E). From the point of view of the promoter, it must be the total number and strengths of the associated TADs that together determine its level of activation. With multiple TADs per transcription factor, efficient synergistic activation may be achieved with a more limited number of factors than if each factor is only carrying single TADs (illustrated in Fig. 11). Whether each of the TADs recruits the same (Fig. 11D) or different co-activators (Fig. 11E) remains to be determined.

Another aspect that remains to be clarified is whether the switch from negative to positive synergy occurs by removal of an obstructive effect of SUMO allowing co-activator recruitment (in Fig. 11 from 11A to 11D/E), or by a change from c-Myb being associated with a multivalent SUMO-binding repressor to being recruiting one or several co-activators (in Fig. 11 from 11B to 11D/E). An indirect argument for the latter is our observation (Fig. 3C) that the helical arrangement of the MREs did not affect the positive synergy observed with c-Myb 2KR (co-activator recruitment), but did affect the low level of SF observed with c-Myb wild-type, arguing that the repressed state of sumoylated c-Myb is more sensitive to geometrical arrangement than the non-

sumoylated active state. This suggests an intermolecular mechanism for keeping c-Myb in a low-activity state. If our current search for a multivalent SUMO-binding repressor is successful, this may clarify the mechanism further.

We realize that v-Myb appears to be a counter example of our model having only one TAD and still displaying a strong synergy. However, v-Myb contains two deletions and several point mutations, which probably affect promoter activation through multiple mechanisms and is therefore difficult to evaluate relative to the current model. A more relevant deletion variant is c-Myb-ΔNRD in which the central TAD is strengthened due to the NRD deletion, but where only one TAD is present. The synergy of this c-Myb deletion is significantly lower than for v-Myb (SF = 1.8 versus 4.8 for v-Myb).

A question worth asking is whether this TAD-model is specific for c-Myb or reflecting a more general mechanism. This remains to be established. It is however worth noticing a few interesting parallels to nuclear receptors (NR). Many NRs have at least two activation domains, the ligand-independent activation function, AF-1, which resides in the N-terminal domain, and the ligand-dependent, AF-2, which is localized in the C-terminal domain. Taking GR as example, the AF1 region (amino acids 108 to 317) is in fact sumoylated (K298 and K313) and is subjected to synergy control. Assuming then that AF-1 may be switched on or off depending sumoylation status, while AF-2 is turned on upon ligand binding, this represents an interesting parallel to c-Myb with the potential to act with different numbers of TADs in a SUMO-controlled manner.

In this study of how SUMO-conjugation restricts the synergy behavior of c-Myb we have discovered a novel switch-property of NRD, providing c-Myb with a potential strong regulatory switch. Future studies will focus on how the activation potential of c-Myb is unleashed through desumoylation.

FOOTNOTES

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REFERENCES

1. Lin, Y. S., Carey, M., Ptashne, M., and Green, M. R. (1990) *Nature* **345**(6273), 359-361
2. Carey, M., Lin, Y. S., Green, M. R., and Ptashne, M. (1990) *Nature* **345**(6273), 361-364
3. Chi, T., Lieberman, P., Ellwood, K., and Carey, M. (1995) *Nature* **377**(6546), 254-257
4. Merika, M., and Orkin, S. H. (1995) *Mol Cell Biol* **15**(5), 2437-2447
5. Merika, M., and Thanos, D. (2001) *Curr Opin Genet Dev* **11**(2), 205-208
6. Guarente, L. (1995) *Trends Biochem Sci* **20**(12), 517-521
7. Adams, C. C., and Workman, J. L. (1995) *Mol Cell Biol* **15**(3), 1405-1421
8. Blau, J., Xiao, H., McCracken, S., O'Hare, P., Greenblatt, J., and Bentley, D. (1996) *Mol Cell Biol* **16**(5), 2044-2055
9. Iniguez-Lluhi, J. A., and Pearce, D. (2000) *Mol Cell Biol* **20**(16), 6040-6050
10. Holmstrom, S., Van Antwerp, M. E., and Iniguez-Lluhi, J. A. (2003) *Proc Natl Acad Sci U S A* **100**(26), 15758-15763
11. Tian, S., Poukka, H., Palvimo, J. J., and Janne, O. A. (2002) *Biochem J* **367**(Pt 3), 907-911
12. Subramanian, L., Benson, M. D., and Iniguez-Lluhi, J. A. (2003) *J Biol Chem*
13. Gill, G. (2005) *Curr Opin Genet Dev* **15**(5), 536-541
14. Hay, R. T. (2005) *Mol Cell* **18**(1), 1-12
15. Muller, S., Ledl, A., and Schmidt, D. (2004) *Oncogene* **23**(11), 1998-2008
16. Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006) *Annu Rev Cell Dev Biol*
17. Ramsay, R. G. (2005) *Growth Factors* **23**(4), 253-261
18. Oh, I. H., and Reddy, E. P. (1999) *Oncogene* **18**(19), 3017-3033
19. Dahle, O., Andersen, T. O., Nordgard, O., Matre, V., Del Sal, G., and Gabrielsen, O. S. (2003) *Eur J Biochem* **270**(6), 1338-1348
20. Bies, J., Markus, J., and Wolff, L. (2002) *J Biol Chem* **277**(11), 8999-9009
21. Britos-Bray, M., and Friedman, A. D. (1997) *Mol Cell Biol* **17**(9), 5127-5135
22. Postigo, A. A., Sheppard, A. M., Mucenski, M. L., and Dean, D. C. (1997) *EMBO J* **16**(13), 3924-3934
23. Burk, O., and Klempnauer, K. H. (1999) *Biochim Biophys Acta* **1446**(3), 243-252
24. Burk, O., Mink, S., Ringwald, M., and Klempnauer, K. H. (1993) *EMBO J* **12**(5), 2027-2038
25. Oelgeschlager, M., Janknecht, R., Krieg, J., Schreek, S., and Luscher, B. (1996) *Embo J* **15**(11), 2771-2780
26. Oelgeschlager, M., Nuchprayoon, I., Luscher, B., and Friedman, A. D. (1996) *Mol Cell Biol* **16**(9), 4717-4725
27. Shapiro, L. H. (1995) *J Biol Chem* **270**(15), 8763-8771
28. Kishi, H., Jin, Z. X., Wei, X. C., Nagata, T., Matsuda, T., Saito, S., and Muraguchi, A. (2002) *Blood* **99**(2), 576-583
29. Seth, A., Gonzalez, F. A., Gupta, S., Raden, D. L., and Davis, R. J. (1992) *J Biol Chem* **267**(34), 24796-24804
30. Saether, T., Berge, T., Ledsaak, M., Matre, V., Alm-Kristiansen, A. H., Dahle, O., Aubry, F., and

Gabrielsen, O. S. (2007) *J Biol Chem* **282**(19), 13994-14005
31. Bonner, J. J. (1991) *Gene* **104**(1), 113-118
32. Andersson, K. B., Kowenz-Leutz, E., Brendeford, E. M., Tygsett, A. H., Leutz, A., and Gabrielsen, O. S. (2003) *J Biol Chem* **278**(6), 3816-3824
33. Cheng, J., Wang, D., Wang, Z., and Yeh, E. T. (2004) *Mol Cell Biol* **24**(13), 6021-6028
34. Andersson, K. B., Berge, T., Matre, V., and Gabrielsen, O. S. (1999) *J Biol Chem* **274**(31), 21986-21994
35. Oelgeschlager, M., Krieg, J., Luscher-Firzlaff, J. M., and Luscher, B. (1995) *Mol Cell Biol* **15**(11), 5966-5974
36. Gabrielsen, O. S., Matre, V., and Bergholtz, S. (2000) Protein-Oligonucleotide interactions. In., John Wiley % Sons Ltd, Chichester
37. Ness, S. A., Marknell, A., and Graf, T. (1989) *Cell* **59**(6), 1115-1125
38. Ording, E., Bergholtz, S., Brendeford, E. M., Jamin, N., and Gabrielsen, O. S. (1996) *Oncogene* **13**(5), 1043-1051
39. Sleeman, J. P. (1993) *Oncogene* **8**(7), 1931-1941
40. Boggio, R., and Chiocca, S. (2006) *Curr Opin Microbiol* **9**(4), 430-436
41. Wysocka, J., and Herr, W. (2003) *Trends Biochem Sci* **28**(6), 294-304
42. Ness, S. A., Kowenz-Leutz, E., Casini, T., Graf, T., and Leutz, A. (1993) *Genes Dev* **7**(5), 749-759
43. Girdwood, D., Bumpass, D., Vaughan, O. A., Thain, A., Anderson, L. A., Snowden, A. W., Garcia-Wilson, E., Perkins, N. D., and Hay, R. T. (2003) *Mol Cell* **11**(4), 1043-1054
44. Yang, S. H., and Sharrocks, A. D. (2004) *Mol Cell* **13**(4), 611-617
45. Ross, S., Best, J. L., Zon, L. I., and Gill, G. (2002) *Mol Cell* **10**(4), 831-842
46. Chen, R. H., and Lipsick, J. S. (1993) *Mol Cell Biol* **13**(7), 4423-4431
47. Dubendorff, J. W., Whittaker, L. J., Eltman, J. T., and Lipsick, J. S. (1992) *Genes Dev* **6**(12B), 2524-2535
48. Sakura, H., Kanei-Ishii, C., Nagase, T., Nakagoshi, H., Gonda, T. J., and Ishii, S. (1989) *Proc Natl Acad Sci U S A* **86**(15), 5758-5762
49. Komatsu, T., Mizusaki, H., Mukai, T., Ogawa, H., Baba, D., Shirakawa, M., Hatakeyama, S., Nakayama, K. I., Yamamoto, H., Kikuchi, A., and Morohashi, K. (2004) *Mol Endocrinol* **18**(10), 2451-2462
50. Miller, A. J., Levy, C., Davis, I. J., Razin, E., and Fisher, D. E. (2005) *J Biol Chem* **280**(1), 146-155

Figure Legends

Figure 1. A SUMO-mediated synergy control (SC) is operating on c-Myb (**A**) Schematic picture of the luciferase reporters used in this study, containing different numbers of Myb responsive elements (MREs) upstream of a core promoter from human *MYC* driving the luciferase reporter gene. (**B**) CV-1 cells were transfected with reporter plasmids containing increasing numbers of MREs (1× - 5×) as indicated and a plasmid encoding c-Myb wild-type or a SUMO-negative c-Myb 2KR. Reporter activation is presented as relative luciferase units (RLU)±SEM. (**C**) Illustration of how we define the synergy factor, SF. (**D,E**) CV-1 cells were transfected with reporters containing one or four Myb responsive elements (1×MRE(GG)-MYC or 4×MRE(GG)-MYC) and plasmids encoding either c-Myb wild-type, c-Myb with both SUMO-conjugation sites mutated from lysine to arginine (2KR), single SUMO-conjugation sites mutated (K503R/K527R) or a 2KR-mutant fused to SUMO-1 as indicated. The results are presented as synergy factor (SF) ± SEM.

Figure 2. The level of synergy is highly dependent on the sumoylation status of c-Myb. CV-1 cells were transfected with reporter plasmids containing one or four MREs (1×MRE(GG)-MYC/4×MRE(GG)-MYC) and (**A**) a plasmid encoding either c-Myb wild-type or a SUMO-negative c-Myb 2KR, with or without the SUMO-protease SENP1 or SENP1 mutant as indicated. (**B**) The same reporter plasmids as above were co-transfected with plasmids encoding either c-Myb wild-type, c-Myb with single SUMO-conjugation sites mutated from glutamate to alanine (E505A, E529A) or with both sites mutated (2EA). The results are presented as SF ± SEM.

Figure 3. The level of synergy is altered in v-Myb, by fusion to virus TAD VP16 and by helical orientation of MREs. CV1 cells were transfected with Myb-responsive reporters containing one or four Myb responsive elements (1×MRE(GG)-MYC or 4×MRE(GG)-MYC) along with a construct encoding (**A**) c-Myb wild-type or the oncogenic version AMV v-Myb. (**B**) The same reporter plasmids as above were co-transfected with either c-Myb wild-type or c-Myb fused to the herpes simplex virus VP16 transactivation domain (amino acid residues 413-488). The results are presented as SF ± SEM. (**C**) The synergy factors were measured using wild-type c-Myb and c-Myb 2KR expression plasmids together with two variants of a luc reporter activated by four MRE differing in the helical phasing of the MREs. In the upper half the pGL4-4×MRE(GG)-MYC reporter was used where the MRE phasing is 10 bp while in the lower half the pGL4-4×MRE(GG)-MYC abab reporter was used where the MRE phasing is 15 bp.

Figure 4. Different c-Myb-mediated synergy on natural chromatin-embedded promoters. Plasmids expressing c-Myb or the sumoylation-negative mutant c-Myb-2KR were transfected into HD-11 cells and total RNA isolated. Activations of the endogenous Myb-target genes *mim*-1, *lysozyme* and *Pdcd4* were measured by quantitative real time PCR using primers specific for each gene, using the chicken *HPRT* as reference gene. The results are presented as relative expression normalized for the *HPRT* expression where the level measured with empty vector is set to 100. The results represent the mean ± SEM of three independent biological assays, each analyzed in triplicate for expression levels.

Figur 5. TSA treatment does not affect SC. (**A**) CV1 cells were transfected with Myb-responsive reporters containing one or four Myb responsive elements (1×MRE(GG)-MYC or 4×MRE(GG)-MYC) along with a construct encoding c-Myb wild-type. 8 hours after transfection, the cells were treated with 100 nM TSA or control (EtOH) for 14 h. The results are presented as SF ± SEM. (**B**) Control of TSA treatment. CV1 cells were transfected with the SNRPN-luc reporter (30) and treated with TSA as described above where indicated. Reporter activations are presented as relative luciferase units (RLU) ± SEM.

Figur 6. SC is not caused by weakening of the DNA-binding by SUMO. COS-1 cell lysates transfected with plasmids coding for c-Myb wild-type (1, 3, 9 µl, lanes 1-3), SUMO-negative c-Myb 2KR (1, 3, 9 µl, lanes 4-6) or c-Myb 2KR fused to SUMO-1 (1, 3, 9 µl, lanes 7-9) were bound to 1×MRE [γ-^{32}P]-labled probe (20 fmol). Complexes were incubated at 25°C for 10 min before

analysis with EMSA (upper panel). A western blot was also performed (lower panel). The amounts of lysate were adjusted to equal concentrations of proteins by western blot with anti-c-Myb (5e11) antibody.

Figure 7. The NRD region of c-Myb can switch from being repressive to be activating. (**A**) CV-1 cells were transfected with 0.2 or 0.4 μg of plasmids expressing Gal4p-DBD fused to c-Myb NRD wt, 2KR, 2EA, SUMO-1 or 2KR-SUMO-1. The reporter output from the *E1b*-driven Gal4p-responsive reporter plasmid (0.2 μg) was normalized to the effect of Gal4p-DBD (0.2 or 0.4 μg), which was set to 100. The results are presented as relative luciferase units (RLU)±SEM. (**B**) The same setup as in (A) but here with Gal-fusions co-transfected with the SUMO-protease SENP1 or a protease-dead SENP1 mutant. The results are presented as fold change calculated from luciferase units in the presence of SENP1/SENP1mut relative to the levels measured when co-transfected with empty vector (Fold change ± SEM).

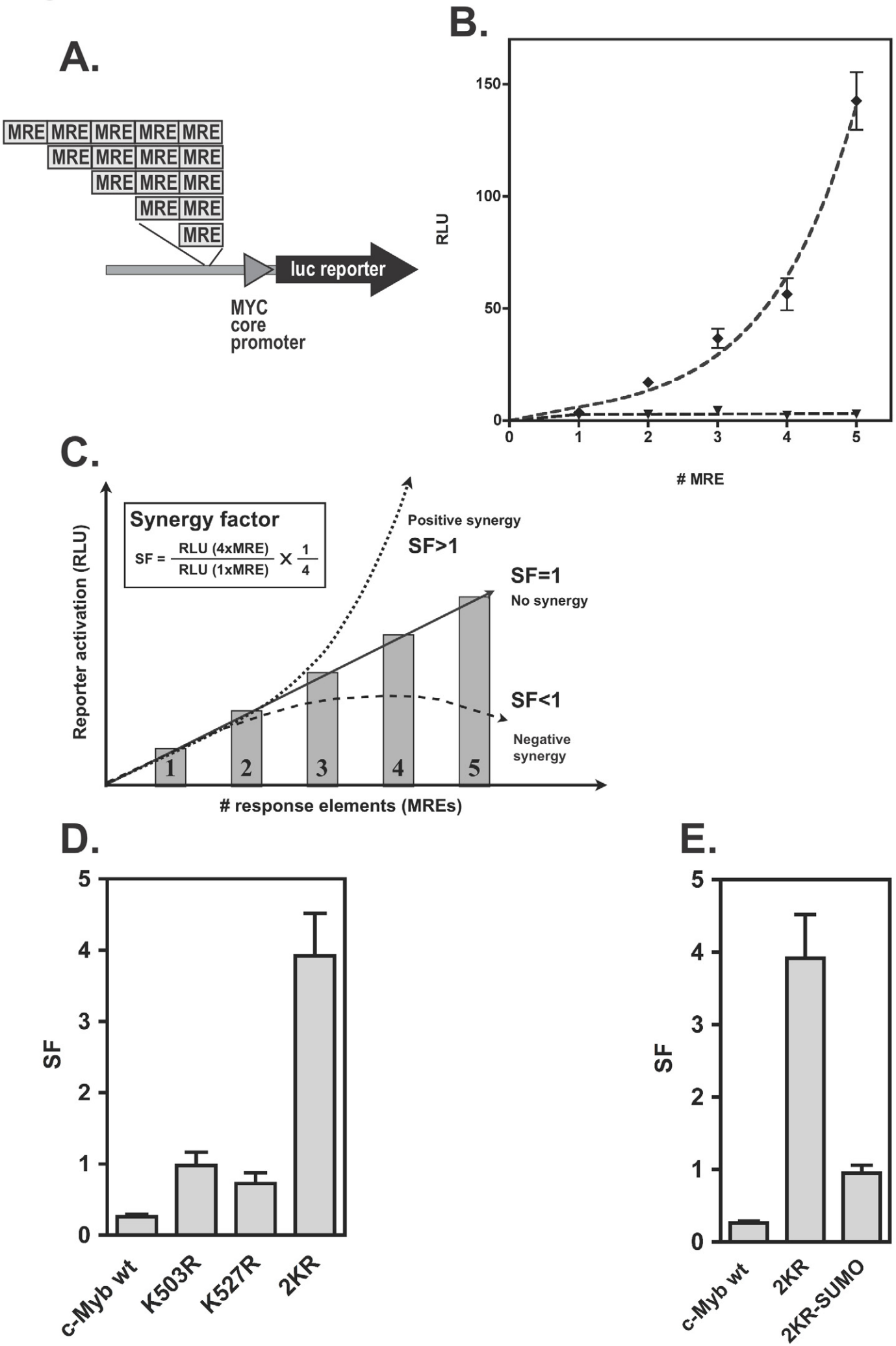
Figure 8. Evidence for two activation domains in c-Myb. CV-1 cells were transfected with a Myb-responsive reporter plasmid containing four MREs (4×MRE(GG)-MYC; 0.2 μg; upper panel) or a reporter plasmid containing three MREs (3×MRE(GG)aab-MYC; 0.2 μg; lower panel) and 0.4 μg of plasmids encoding either full-length c-Myb, its SUMO-conjugation negative mutant c-Myb-2KR, or a deletion variant c-Myb ΔNRD. The results are presented as relative luciferase units (RLU) ± SEM.

Figure 9. Evidence for two activation domains in c-Myb. CV-1 cells were transfected with a Myb-responsive reporter plasmid containing three MREs (3×MRE(GG)aab-MYC; 0.2 μg) and plasmids encoding full-length c-Myb, or a variant with the central transactivation domain deleted (c-Myb-ΔT1) or the latter also harboring the 2KR mutation abolishing SUMO-conjugation (c-Myb-ΔT1-2KR; 0.4 μg). The results are presented as relative luciferase units (RLU)±SEM.

Figure 10. The importance of the number of transactivation domains for level of synergy. CV-1 cells were transfected with reporter plasmids containing one or four MREs (1×MRE(GG)-MYC or 4×MRE(GG)-MYC) and an effector plasmid encoding one of the following c-Myb variants: c-Myb, c-Myb-ΔNRD encoding amino acids 1-443, the SUMO-conjugation negative mutant c-Myb-2KR and the latter fused C-terminally to VP16. The results are presented as SF ± SEM.

Figure 11. A model for SUMO-mediated TAD repression and synergy control. (**A**) A model for the repressed state where SUMO-conjugation disrupts the interaction with the co-activator. (**B**) A model for the repressed state where multiple SUMO-conjugated factors recruit a multivalent co-repressor. (**C**) Traditional concept of synergy mediated by joint recruitment of a multivalent co-activator. (**D**) Current model for the activated state where multiple TADs (depicted as stars) per transcription factor lead to more efficient recruitment of a multivalent co-activator. (**E**) A variant of the model above (in Figure 11D) where different TADs may interact with different co-activators.

Figure 1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MRE MRE MRE
MRE MRE
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luc reporter
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core
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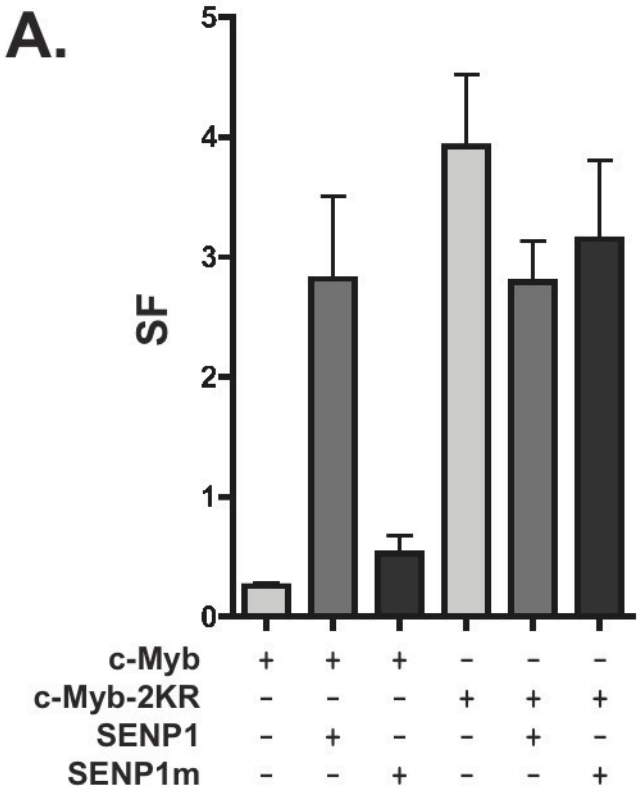
B.
RLU
150
100
50
0
0 1 2 3 4 5
MRE
C.
Synergy factor
SF = RLU (4xMRE) / RLU (1xMRE) X 1/4
Positive synergy
SF>1
SF=1
No synergy
SF<1
Negative
synergy
Reporter activation (RLU)
1 2 3 4 5
response elements (MREs)
D.
SF
5
4
3
2
1
0
c-Myb wt
K503R
K527R
2KR
E.
SF
5
4
3
2
1
0
c-Myb wt
2KR
2KR-SUMO

Figure 2


A.
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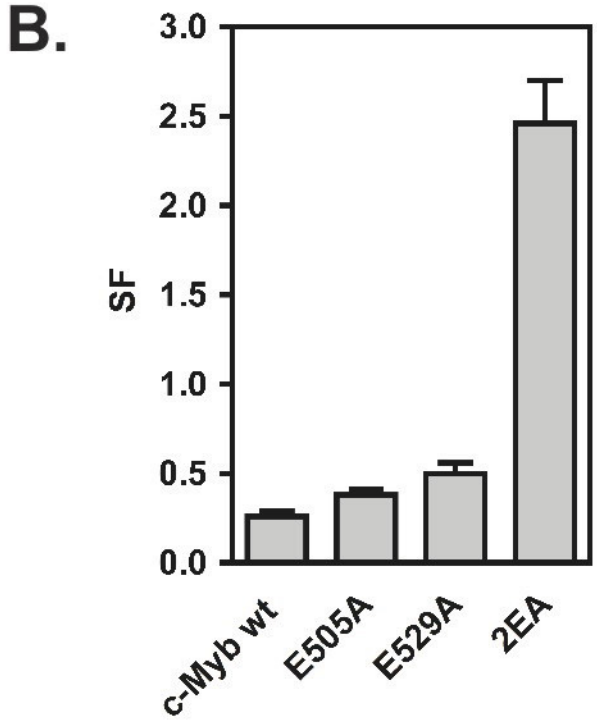
B.
SF
3.0
2.5
2.0
1.5
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0.5
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c-Myb wt
E505A
E529A
2EA

Figure 3

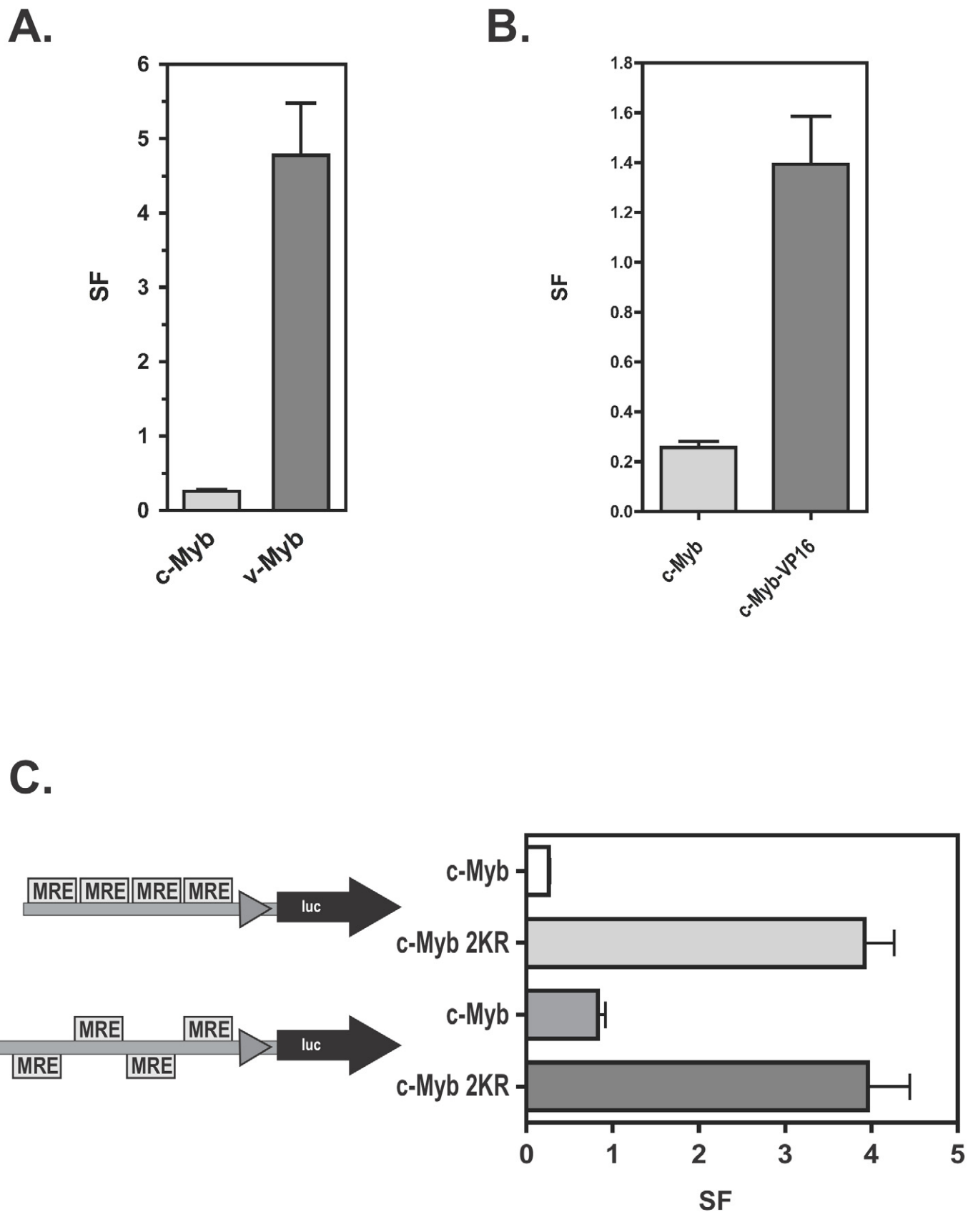
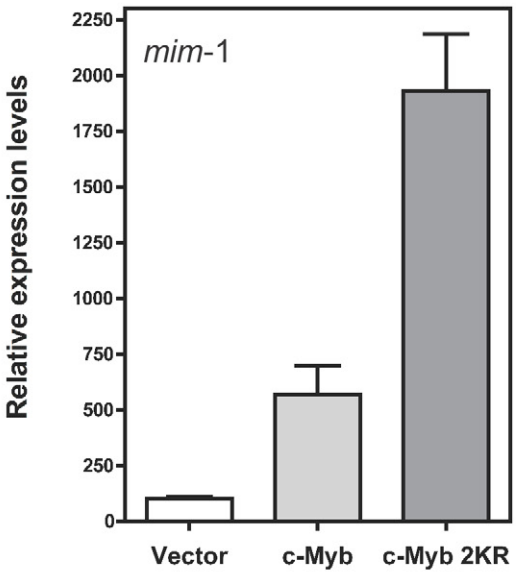
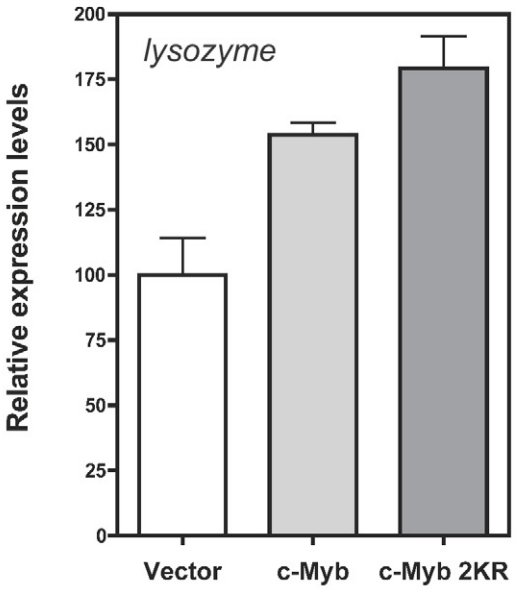

A.
SF
6
5
4
3
2
1
0
c-Myb
v-Myb
B.
SF
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
c-Myb
c-Myb-VP16
C.
MRE
MRE
MRE
MRE
luc
MRE
MRE
MRE
MRE
luc
c-Myb
c-Myb 2KR
c-Myb
c-Myb 2KR
0
1
2
3
4
5
SF

Figure 4

A.


mim-1
Relative expression levels
2250
2000
1750
1500
1250
1000
750
500
250
0
Vector
c-Myb
c-Myb 2KR

B.


lysozyme
Relative expression levels
200
175
150
125
100
75
50
25
0
Vector
c-Myb
c-Myb 2KR

C.

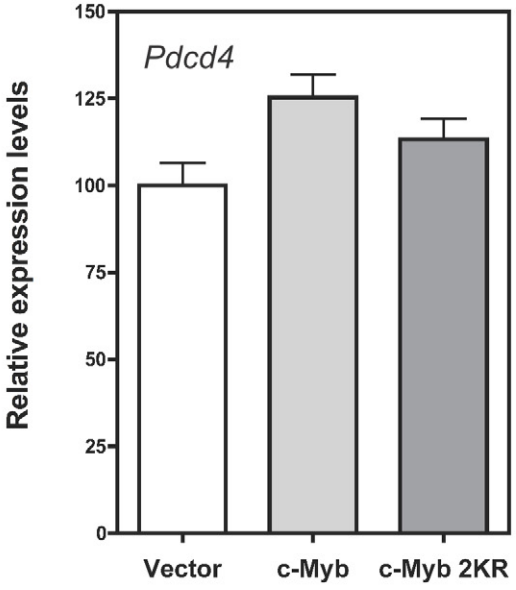
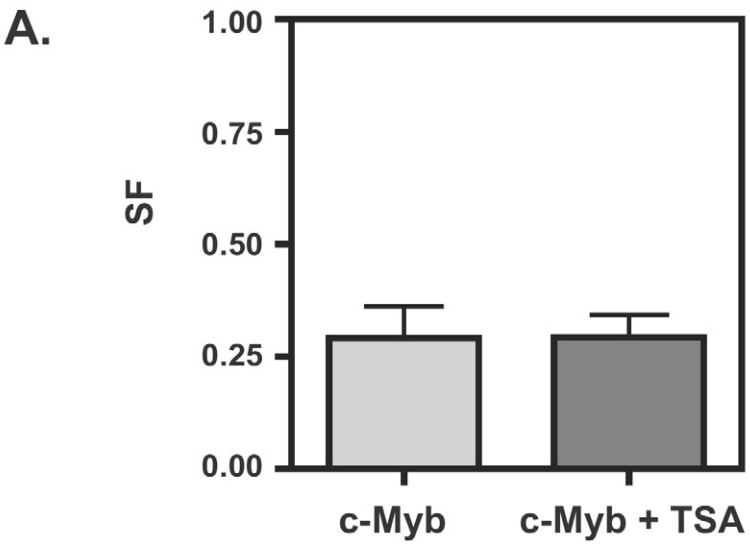

Pdcd4
Relative expression levels
150
125
100
75
50
25
0
Vector
c-Myb
c-Myb 2KR

Figure 5


A.
1.00
0.75
0.50
0.25
0.00
SF
c-Myb
c-Myb + TSA

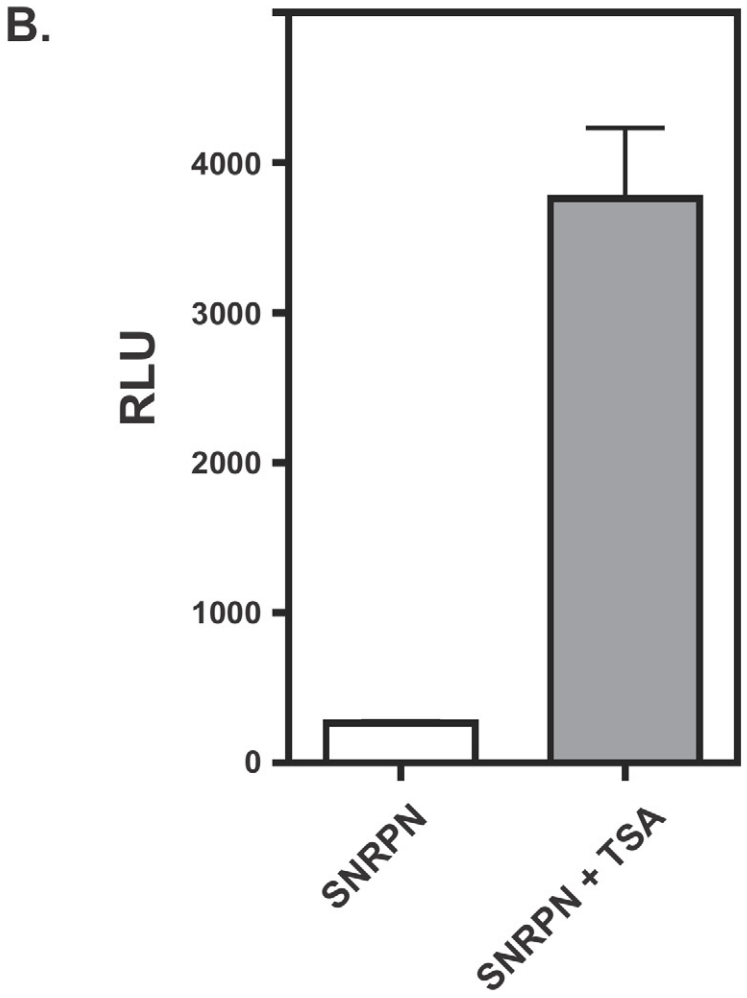

B.
4000
3000
2000
1000
0
RLU
SNRPN
SNRPN + TSA

Figure 6

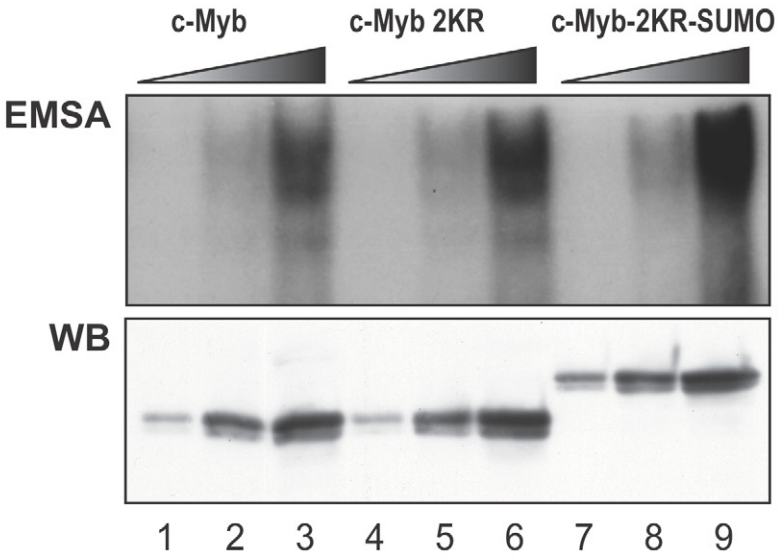
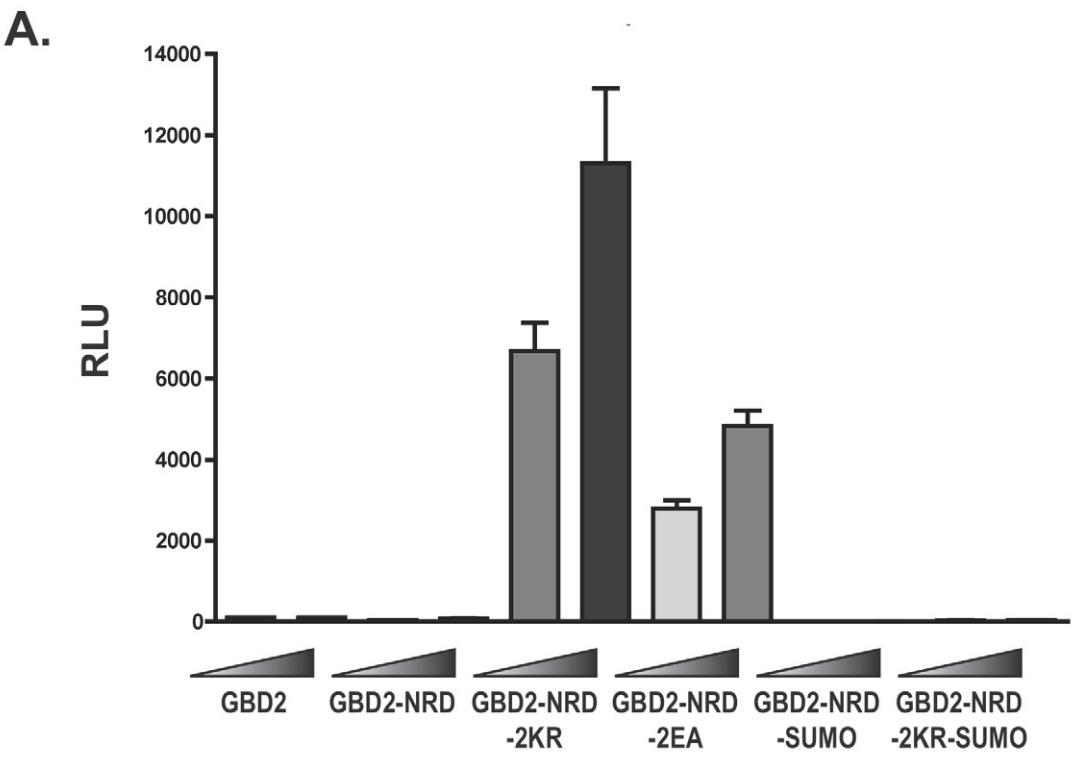


Figure 7


A.
RLU
14000
12000
10000
8000
6000
4000
2000
0
GBD2
GBD2-NRD
GBD2-NRD -2KR
GBD2-NRD -2EA
GBD2-NRD -SUMO
GBD2-NRD -2KR-SUMO

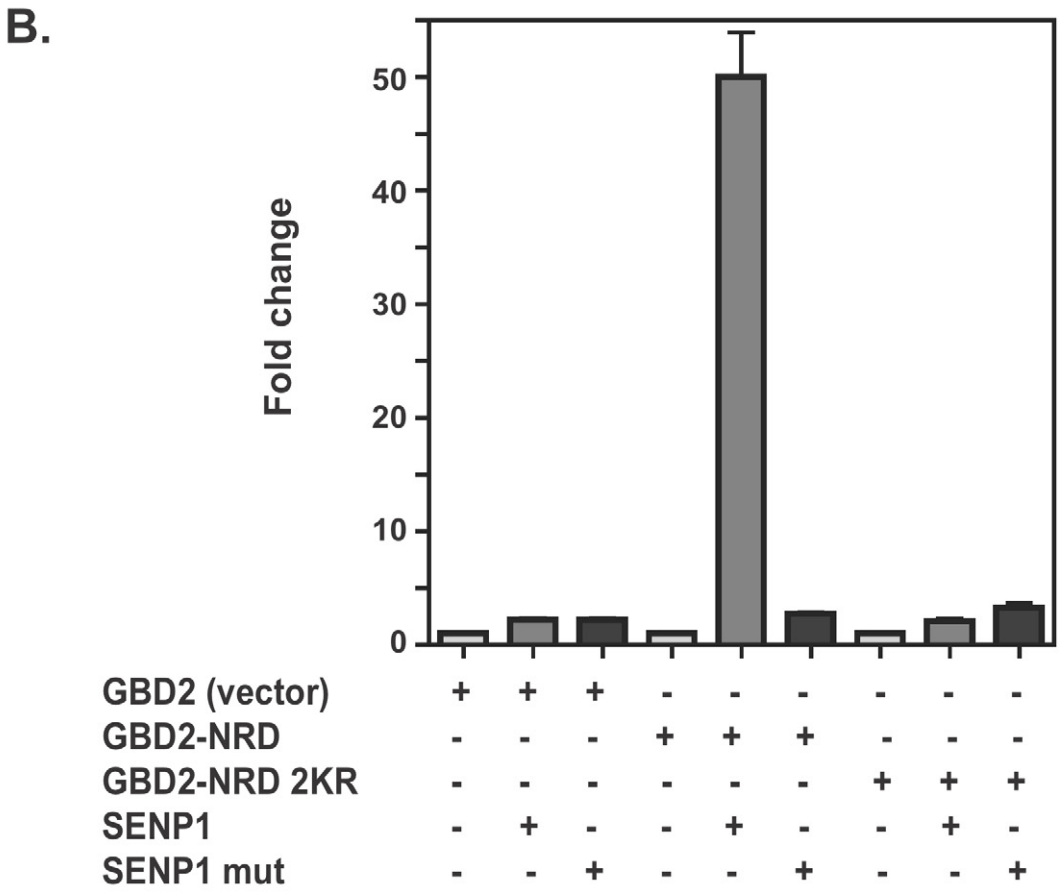

B.
Fold change
50
40
30
20
10
0
GBD2 (vector) + + + - - - - - -
GBD2-NRD - - - + + + - - -
GBD2-NRD 2KR - - - - - - + + +
SENP1 - + - - + - - + -
SENP1 mut - - + - - + - - +

Figure 8

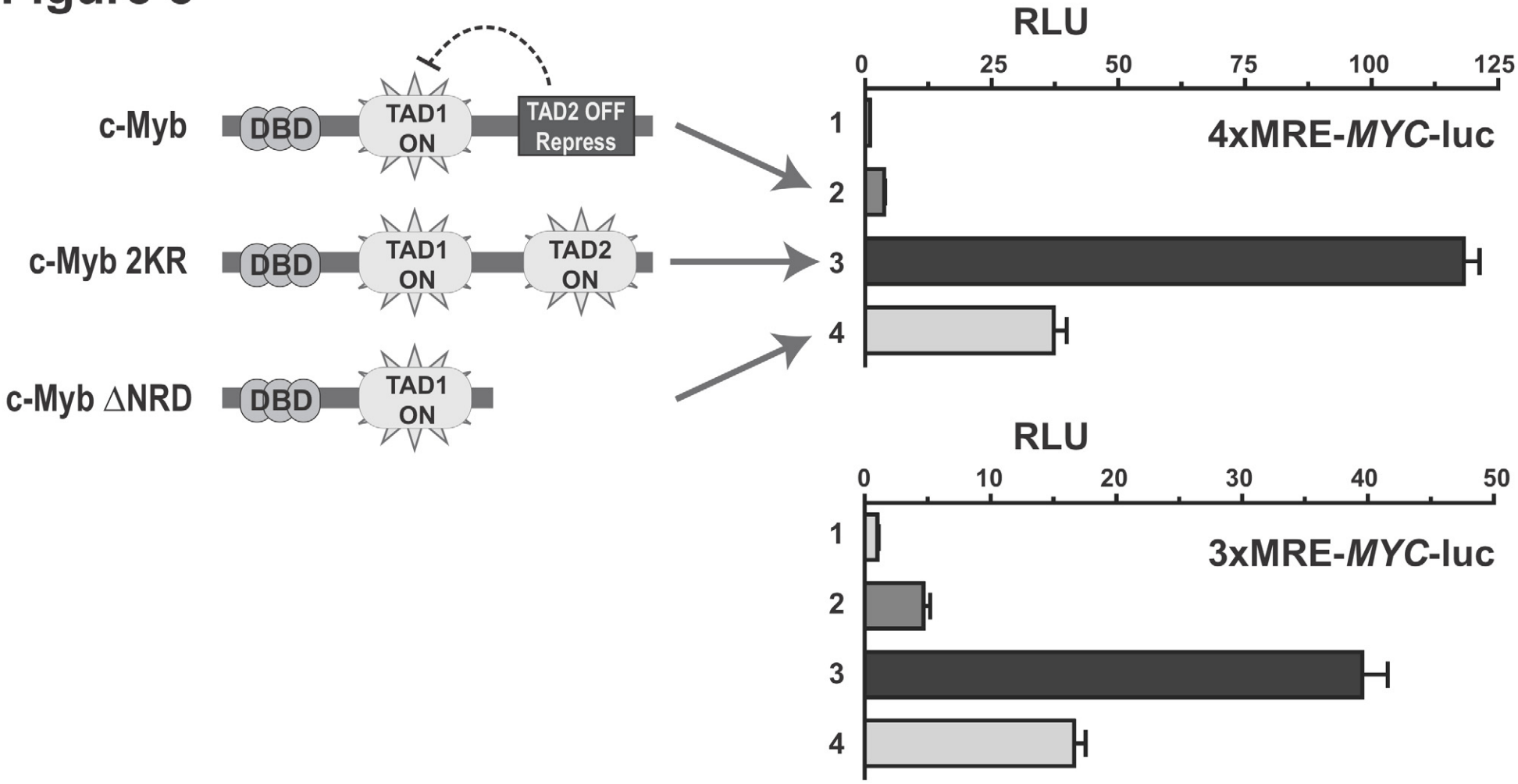

c-Myb
DBD
TAD1 ON
TAD2 OFF Repress
c-Myb 2KR
DBD
TAD1 ON
TAD2 ON
c-Myb ΔNRD
DBD
TAD1 ON
RLU
0
25
50
75
100
125
1
2
3
4
4xMRE-MYC-luc
RLU
0
10
20
30
40
50
1
2
3
4
3xMRE-MYC-luc

Figure 9

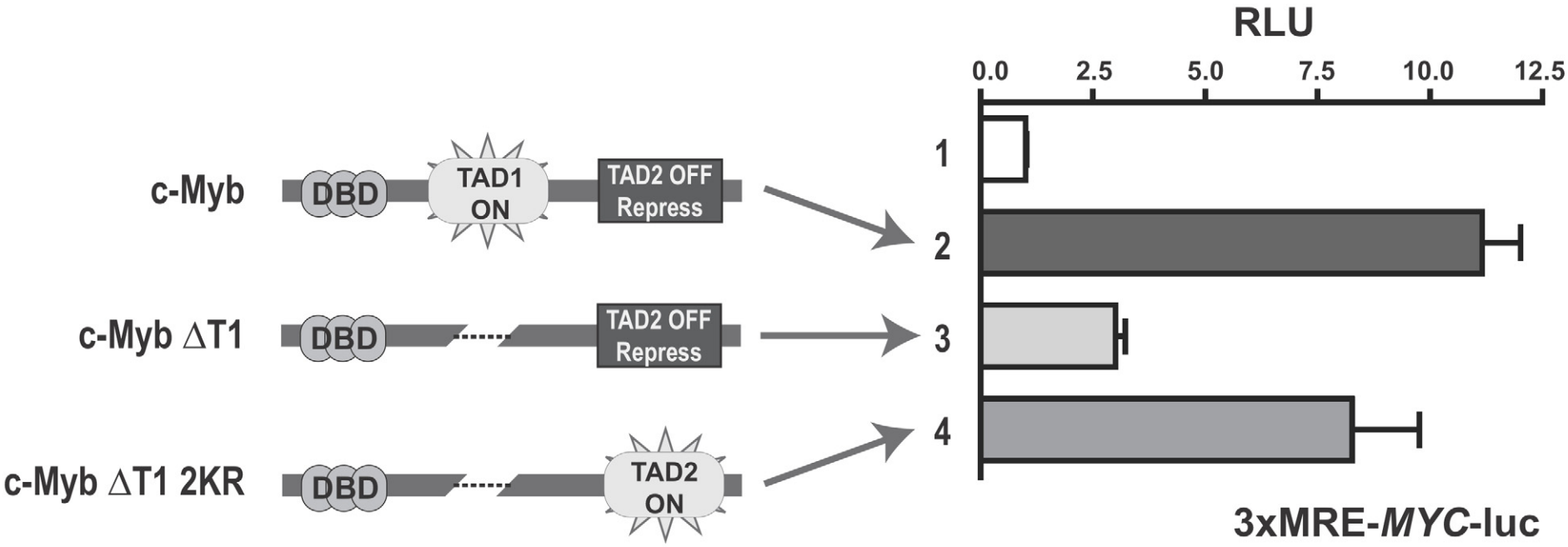

c-Myb
DBD
TAD1 ON
TAD2 OFF Repress
c-Myb ΔT1
DBD
TAD2 OFF Repress
c-Myb ΔT1 2KR
DBD
TAD2 ON
RLU
0.0
2.5
5.0
7.5
10.0
12.5
1
2
3
4
3xMRE-*MYC*-luc

Figure 10

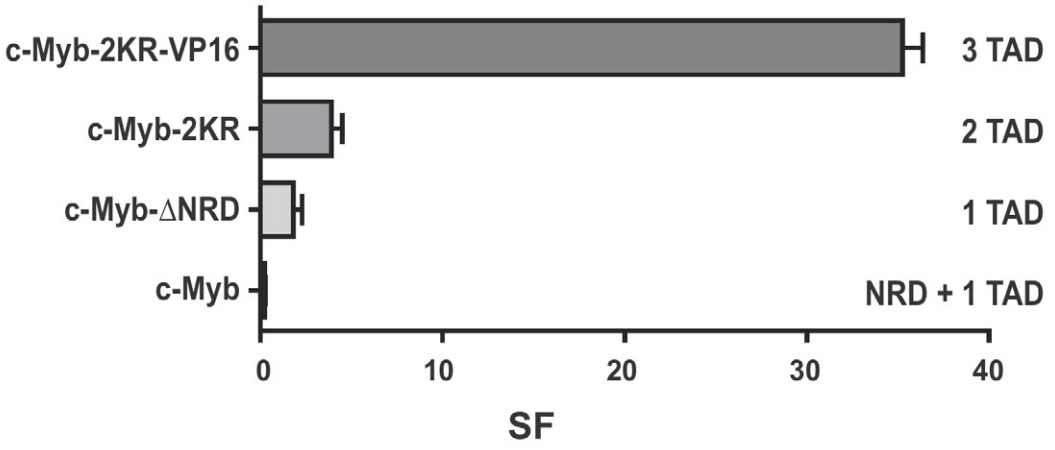
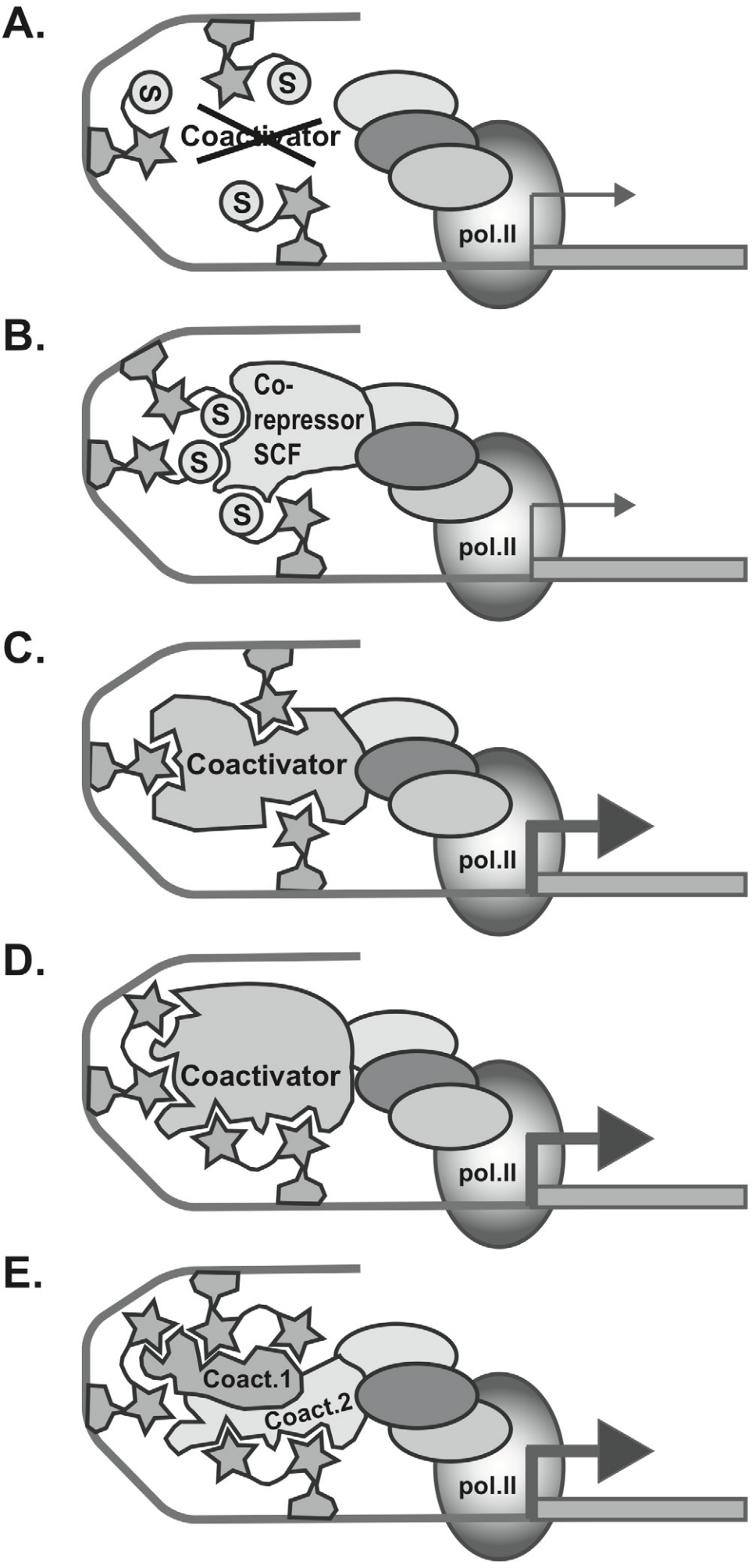

c-Myb-2KR-VP16
c-Myb-2KR
c-Myb-ΔNRD
c-Myb
3 TAD
2 TAD
1 TAD
NRD + 1 TAD
0
10
20
30
40
SF

Figure 11


A.
S
S
S
Coactivator
pol.II
B.
Co-
repressor
SCF
S
S
S
pol.II
C.
Coactivator
pol.II
D.
Coactivator
pol.II
E.
Coact.1
Coact.2
pol.II

Paper IV

IV

A FUNCTIONAL SUMO-BINDING MOTIF IN THE TRANS-ACTIVATION DOMAIN OF c-MYB REGULATES ITS ACTIVITY*

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AMV v-*myb*, an oncogenic version of c-*myb*, encodes a protein deleted both N- and C-terminally as well as harbouring 10 point mutations. While the N-terminal deletion and mutations in the DNA-binding domain (DBD) influence DNA- and histone-binding, the C-terminal deletion removes most of the negative regulatory domain (NRD) and eliminates SUMO-conjugation. This ensures a high transactivational potential in v-Myb. Based on reports of a consensus SUMO-binding motif (SBM) we analysed the c-Myb sequence and found that it contains two putative SBMs; one in the DBD and one in the transactivation domain (TAD). Remarkably, both SBMs are found mutated in AMV v-Myb. Pull-down and reporter assays showed that the SBM in the TAD of c-Myb (V_{267}NIV; SMB2) is functional. This motif is necessary for c-Myb to be able to interact non-covalently with SUMO, preferentially SUMO-2/3. Destroying the SUMO-binding properties of c-Myb by mutation of SBM2 resulted in a large increase in the trans-activation potential. This enhancement was not due to impaired SUMO-conjugation or synergy control, but most probably to lost interaction with an unknown repressive partner. Analysis of combined mutations argued against intramolecular repression, caused by SUMO conjugated to c-Myb NRD, while activation induced by overexpression of conjugation-defective SUMO argued in favour of a sumoylated repressor being involved. In search for an intermolecular mechanism we focused on the Promyelocytic Leukemia (PML) protein, previously shown to bind c-Myb. However, confocal imaging of PML and c-Myb showed that c-Myb is recruited to PML nuclear bodies (PML-NBs) in a SUMO contact-independent manner. Most likely PML is therefore not the repressive partner itself. Interestingly, the SUMO-independent recruitment of c-Myb to PML-NBs did not correlate with functional data showing that c-Myb wild-type is activated by ectopic PML expression, while the SUMO-binding and sumoylation-negative mutants have lost most of this potential. We therefore hypothesize that c-Myb can be relieved from sumoylated and SUMO-binding repressive factors through their sequestering in PML-NBs, and that this loss of co-repression is mimicked by the SUMO contact-negative mutations.

Through the last decade, studies within the field of SUMO (Small Ubiquitin-like modifier) have continuously revealed new and fascinating roles for this small nuclear peptide, and the number of targeted proteins is constantly growing. Despite the diversity of targets, there seems to be some common themes in functional effects: Covalent conjugation of SUMO to nuclear factors mainly suppress their activity or synergistic potential, alter their localization and interaction repertoire, or increase their stability [2-5]. The three best studied SUMO isoforms; SUMO-1, -2, and -3, are all conjugated to lysine in the consensus sequence ψKXE which often has additional acidic residues located directly C-terminally of this motif [6, 7]. SUMO-2 and -3, which share 98 % identity, exist predominantly as unconjugated proteins, but have been shown to be incorporated into high molecular weight complexes as a result of oxidative, heat or genotoxic stress [8]. This contrasts SUMO-1, which shares ~50 % identity with SUMO-2/3 and does not seem to exist in any large detectable free pool, but must be de-conjugated and conjugated quite rapidly [8]. Together with other Ubiquitin-like proteins such as ISG15, NEDD8, FAT10 and Ubiquitin itself, SUMO participates in a dynamic inter-play with the targeted proteins (reviewed in [5]). The molecular signals, however, determining the spatial and temporal pattern of

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sumoylation versus de-sumoylation have yet to be identified.

With the increasing number of sumoylated targets, and the common assumption that the effects of SUMO have to be mediated through protein interactions, the identification of a protein motif for non-covalent SUMO-binding was awaited. In 2004 Song *et al*. showed using NMR that a small hydrophobic patch, V/I-X-V/I-V/I, was the minimal motif needed for SUMO-interaction [9]. This only partly fitted with a motif proposed earlier (h-h-X-S-X-S/T-a-a-a) by Minty *et al*. [10]. However, with a study in yeast [11] and the work of Hecker *et al*. [12] the suggested consensus sequences were harmonized; V/I-X-V/I-V/I-a-a-a. Furthermore, the motif was demonstrated to be able to bind SUMO when reversed; a-a-a-V/I-V/I-X-V/I/L [12, 13]. In mammalian proteins the hydrophobic core allows for SUMO-binding, while negative charged amino acids surrounding the core determine the specificity regarding which of the SUMO-isoforms will bind. SUMO-1 seems to be more dependent on such an acidic stretch for efficient binding, while SUMO-2/3 can bind in its absence [12].

The discovery of SUMO-binding motifs (SBMs) has provided new insight into the interplay between sumoylation and SUMO-binding, with the tumour suppressor PML as one of the best examples. PML contains both sumoylation and SUMO-binding motifs, and a recent paper showed how both motifs must be intact to form PML nuclear bodies (PML-NBs) [14]. Furthermore, the presence of several unoccupied SUMO-moieties and SBMs on the surface of the PML NBs allows for sumoylated and/or SBM-containing factors to be recruited to these bodies [14]. For the co-repressor Daxx an internal SBM has been shown to be crucial for targeting it to PML-NBs [15], while the base-excision repair enzyme Thymine DNA Glycosylase (TDG) has to be desumoylated to be able to translocate to PML-NBs, probably because the SUMO-moiety occludes the SBMs in TDG through binding [16].

Another nuclear factor that associates with PML NBs is c-Myb [17]. c-Myb is a sequence-specific transcription factor that controls proliferation and differentiation of early hematopoietic progenitor cells, as well as regulating similar processes in other tissues [1, 18]. c-Myb becomes sumoylated in its negative regulatory domain at two sites, by both SUMO-1 and SUMO-2/3 [19-21]. This leads to a severe drop in the activity of c-Myb [20, 21]. In a recent paper we have shown that this drop in activity is mainly due to enforced SUMO-control of the synergistic potential of c-Myb and the silencing of a TAD-function in NRD [22]. When c-Myb is desumoylated at both conjugation sites the full synergistic potential of the transcription factor is unleashed and c-Myb operates with two TADs. In v-Myb, the oncogenic version of c-Myb, both SUMO-conjugation sites are deleted, hence no synergy control (SC) can be imposed by SUMO. A strategy to by-pass SUMO-conjugation and escape SC may be a common theme in oncogenic activation [22].

In this paper we show that the transactivation potential of c-Myb is modulated not only through SUMO-conjugation and SC, but also through non-covalent SUMO-binding. We have identified a functional SBM in the N-terminal part of the central transactivation domain. This motif is necessary for non-covalent interaction of SUMO with c-Myb, which preferentially binds SUMO-2/3. When mutated it induces an increase in c-Myb transactivational activity. This increase is not due to impaired SUMO-conjugation, lost synergy control or SUMO-mediated intramolecular interactions being broken, but rather lost repression in *trans*. Confocal imaging show that c-Myb is recruited to PML nuclear bodies in a SUMO contact-independent manner. At the same time co-transfection with PML activate c-Myb. However, when the SUMO-contact motifs (SUMO-binding and conjugation) are mutated, c-Myb looses this potential. This is probably due to relieve of co-repression imposed on c-Myb by SUMO-binding and/or sumoylated factors, being sequestered by PML-NBs.

EXPERIMENTAL PROCEDURES

Plasmids - The mammalian expression vectors pCIneo-hcM-HA and pCIneo-hcM-HA-2KR (encoding wild type and sumoylation-deficient c-Myb, respectively) have been described [20]. The plasmids encoding the different SBM mutants (pCIneo-hcM-HA A_{103}AEA, L106H, A_{267}NAA and I_{267}NII) were generated using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) on subfragments of human c-Myb. The "double mutant" pCIneo-hcM-HA A_{267}NAA 2KR was made by subcloning the *Bgl*II-*Not*I fragment

from pCIneo-hcM-HA 2KR into pCIneo-hcM-HA A_{267}NAA.

The 3×FLAG tagged human c-Myb, pCIneoB-3FLAG-hcM, was made by subcloning human c-*MYB* from pCIneo-hcM into pCIneoB-3FLAG (described earlier [23]) between *Xho*I and *Not*I. pCIneoB-3FLAG-hcM[1-409], encoding human c-Myb and covering amino acid residues 1-409, was made by PCR amplification of the DBD-FAETL part of human c-Myb, and subcloning this fragment into pCIneoB-3FLAG between *Sal*I and *Not*I. The pCIneoB-3FLAG-hcM[1-409] A_{103}AEA, L106H, A_{267}NAA and I_{267}NII were made by subcloning the *Xho*I-*Eco*RI fragment from the corresponding full-length construct into pCIneoB-3FLAG-hcM[1-409]. The pCIneoB-3FLAG-AMV encoding a FLAG-tagged AMV v-Myb protein was made by subcloning the *Xho*I-*Not*I fragment from pCIneo-AMV (described earlier [24]) into pCIneoB-3FLAG-hcM[1-409], replacing hcM[1-409].

The mammalian expression vectors for Gal4p-DBD fused to c-Myb TAD wild-type and A_{267}NAA, covering amino acid residues 259 to 337, were made by PCR amplification of pCIneo-hcM-HA wild-type and A_{267}NAA and subcloning this fragment into pCIneoB-GBD2 (described earlier [23]) between *Sal*I and *Not*I.

The conjugation-deficient SUMO-1 and SUMO-2 expression plasmids, pCIneo-hSUMO1-1G and pCIneo-hSUMO2-1G, were made by PCR amplification of human SUMO-1 (amino acid residues 1-96) and SUMO-2 (amino acid residues 1-91) and subcloning these in pCIneo between *Xho*I and *Not*I. These plasmids encode SUMO-1 and -2 mutants that only retain the first C-terminal Gly of the Gly-Gly motif.

The *TRHR* reporter, pGL2b-TRHR-1250 covering the area -1250 to +1 from the thyrotropin-releasing hormone receptor promoter has been described [25], as well as the *SNRPN* driven Gal4p-responsive luciferase reporter, pGL3b-5GRE-SNRPN [23]. The pGL4b-1×MRE(GG)-MYC, pGL4b-3×MRE(GG)-MYC-aab and pGL4b-4×MRE(GG)-MYC-aaaa reporters were also previously described [22]. All cloned fragments generated by PCR or by oligo-insertion were verified by sequencing. Primer sequences are available upon request.

The p300 expressing mammalian vector pCMVβ-NHA-p300 was a kind gift from Prof. D. Livingston and has been described previously [26], while the vector expressing PML IVa [27] as well as the GST-SUMO fusion expressing plasmid pGEX-2TK-SUMO1 [28] were kindly provided by Prof. G. Del Sal. The pGEX-4T3-hSUMO2 was received as a gift from Prof. RT. Hay and has been described [29], while the pCMV-T7-mPIASy vector [30] was a kind gift from Prof. R. Grosschedl.

Protein expression, GST pull-down assay and in vitro translation – GST and GST-SUMO fusion proteins were expressed in *E. coli* as previously described [31]. GST fusion proteins pre-bound to glutathione-Sepharose beads (GE Healthcare) were incubated over night at 4 ºC with 300 µl total cell extract representing 1×10^6 transfected COS-1 cells lysed in 100 µl Lysis-buffer (150 mM NaCl, 0.6 % Triton X-100, 10 % Glycerol 50 mM Hepes, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 10 µM $ZnCl_2$, 0.1 % β-Mercaptoethanol and Protease Inhibitor cocktail (Roche)), and diluted 1:2 in the same buffer without Triton X-100, to a final concentration of 0.2 % Triton X-100. Alternatively, proteins were *in vitro* translated and [^{35}S]-labelled using TNT Quick Coupled Transcription/Translation Systems (Promega) and diluted in the same buffer as above. Beads were washed two times in 500 µl Interaction buffer (150 mM NaCl, 0.2 % Triton X-100, 10 % Glycerol 50 mM Hepes, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 10 µM $ZnCl_2$, 0.1 % β-Mercaptoethanol and Protease Inhibitor cocktail (Roche)) for 5 min at 4 ºC with rotation, and the proteins eluted in 75 µl 3×SDS loading buffer. Proteins were separated by SDS-PAGE and detected with immunoblotting.

Cell culture and transfection, luciferase assays and immunoblotting - COS-1 and CV-1 cells were grown as described [24]. Both cell lines were transiently transfected with the indicated plasmids using FuGENE6 (Roche). For the luciferase assays transfected CV-1 cells (24-well trays; 2×10^4 cells/well) were harvested 24 hours after transfection and lysed in Passive Lysis Buffer (Promega). Luciferase assays were performed in triplicate using Luciferase Assay Reagent (Promega), and data from at least three independent transfection experiments are presented. In parallel transfected CV-1 cells were lysed in NP-40 buffer and subjected to immunoblotting. For the assessment of SUMO-conjugation of c-Myb mutants transfected CV-1 cells (6-well trays;

1.2×10^5 cells/well) were harvested 24 hours after transfection, scraped in ice cold PBS, and lysed directly by sonication in SDS-PAGE sample buffer.

Antibodies - For immunoblot detection the following antibodies were used; rabbit anti-HA antibody (H 6908 Sigma), mouse anti-FLAG M2 monoclonal antibody (F3165, Sigma), anti-mouse IgG-HRP (NA931, GE Healthcare) and anti-rabbit IgG-HRP (NA934, GE Healthcare). As immunofluorescence antibodies we used rabbit anti-HA (H 6908 Sigma) and mouse anti-PML (sc-966 Santa Cruz). Alexa Fluor 488 goat anti-rabbit IgG(H+L) and Alexa Fluor 633 goat anti-mouse IgG1 (γ1) (Molecular Probes) were used as secondary antibodies.

Immunofluorescence and confocal laser scanning microscopy - 1.8×10^4 CV-1 cells were plated out in 24-well trays containing cover-slips and transfected 24 hours later with the indicated plasmids at a total of 0.6 μg DNA. 24 hours after transfection cells were washed in PBS. Cells were fixed and permeabilized with 4 % formaldehyde for 10 min and ice cold methanol for 2 min. Samples were washed three times for 5 min in PBS containing 0.1 % Tween 20 and then blocked for 30 min with 2 % BSA in PBS with 0.1 % Tween 20, followed by incubation with primary antibodies diluted 1:50 in the blocking solution for 45 min. The samples were then washed three times as described above, and incubated with secondary antibodies diluted 1:100 in 2 % BSA in PBS with 0.1 % Tween 20 for 30 min. Samples were washed three times again and incubated with Hoechst 33258 (Sigma-Aldrich) for 20 min to visualize DNA. After three washes the cover-slips were placed on microscope slides using mounting medium (Dako). Cells were examined using a FluoView laser scanning system from Olympus. Images from the different channels were collected sequentially to prevent bleed through.

RESULTS

c-Myb contains two putative SUMO binding motifs which are both mutated in AMV v-Myb

The C-terminal deletion in AMV v-Myb, an oncogenic version of c-Myb, eliminates the negative regulatory domain (NRD), making AMV v-Myb behave like an activated form of c-Myb in transactivation assays. Lost SUMO-conjugation plays a key role here [20]. We recently came to realize that loss of SUMO-conjugation sites is not the only oncogenic alteration in v-Myb that may relate to SUMO-biology. Based on reports of a consensus SUMO-binding motif (SBM; aaa-V/I-V/I-X-V/I/L and V/I-X-V/I-V/I-aaa) [9, 12, 13], we analysed the c-Myb sequence and found that it contains two putative SBMs; one in the R2 repeat in the DNA-binding domain (termed SBM1), and one in the N-terminal end of the transactivation domain (termed SBM2; Fig 1*A* and *B*). Remarkably, both sites are mutated in v-Myb, as illustrated in Fig 1*B*. In fact, three of the ten oncogenic mutations in v-Myb are localized to the putative SBMs. Hecker *et al*. have shown that the specificity of non-covalent SUMO-1 versus SUMO-2 binding lies in a stretch of negatively charged residues located directly N- or C-terminally of the core SBM motif [12]. The fact that only SBM1 has such neighbouring residues implies that if functional, the c-Myb SBM1 would be a SUMO-1 binding motif, while SBM2 mainly would bind SUMO-2/3.

Mutating the SBM in c-Myb TAD derepress c-Myb and makes it super active.

To investigate whether mutations in these potential SMBs would influence c-Myb activity and thereby give a hint to whether or not these sites are of functional importance, we made a set of mutants aiming to abrogate SUMO-binding (outlined in Fig 1*C*). The mutants L106H and I_{267}NII were made to mimic the mutations found in v-Myb SBM1 and SBM2, respectively. It may be noticed that the sequence change in SBM2 introduced in AMV v-Myb is not expected to abolish SUMO-binding. Hence, since only the L106H mutation seemed to represent a deviation from the SBM consensus, the mutations A_{103}AEA (wild type: V_{103}IEL) and A_{267}NAA (wild type: V_{267}NIV) were made to ensure a complete elimination of any binding of SUMO to the SBMs, given that one or both of the SBMs were functional.

We then performed effector-reporter assays in CV-1 cells using the c-Myb responsive *3×MRE(GG)-MYC* luciferase reporter [22]. As can be seen in Fig 2*A*, the SBM1 mutant L106H apparently had no effect in this system, while the A_{103}AEA appeared to have a slight negative influence on c-Myb-mediated transactivation. On the other hand the A_{267}NAA SBM2 mutant had a dramatic effect and activated, i.e. derepressed, c-Myb more than 13-fold. The activity of this mutant closely

resembled that of the SUMO-conjugation negative 2KR mutant [20], which we included as a positive control (Fig 2*A*). The v-Myb mimicking mutation I_{267}NII had no such derepression effect; it rather lowered the c-Myb activity slightly. These results were also verified on another reporter harbouring the c-Myb responsive *TRHR* promoter (data not shown). As can be seen in Fig 2*B*, none of these effects can be attributed to mutation-induced changes in expressional levels or protein stability. It therefore appears that that the V_{267}NIV motif in c-Myb TAD has a strong repressive function, possibly mediated through the binding of SUMO, and that the A_{267}NAA mutant abrogates this repression.

Human c-Myb binds SUMO

If c-Myb contains a functional SBM, the protein must show some affinity for SUMO. However, since SUMO in general binds to SBMs with affinities in the micromolar range [12], demonstrating interactions may be technically difficult. To examine whether c-Myb is able to bind SUMO, we tried to pull out c-Myb from COS-1 lysates using GST-SUMO-1 and SUMO-2. Until date this is one of the most stringent ways of detecting SUMO-binding, allowing both endogenous SUMO and SUMO-binding factors to compete for epitopes. To avoid the potential problem of SUMO conjugated to c-Myb interfering with any SUMO binding sites we used a shorter version of human c-Myb (spanning amino acid residues 1-409) where the NRD (harbouring the SUMO-conjugation motifs) had been deleted. As can be seen in Fig 3A, c-Myb did interact with SUMO under these conditions, although weakly as expected. Furthermore, c-Myb seemed to bind more efficiently to SUMO-2 than to SUMO-1 (Fig 3A).

Knowing that the shorter version of c-Myb was able to bind SUMO, we then tested if the same held true for full-length c-Myb. Using the same experimental setup as above, we found that GST-SUMO-2 was able to interact with full-length c-Myb as well (Fig 3*B*). The two lower bands seen with full-length c-Myb are the result of some unspecific C-terminal degradation. Nevertheless, this shows that c-Myb binds to SUMO-2, and that the interacting motif is exposed and available when full-length c-Myb is expressed and folded *in vivo*.

Only the putative SBM in c-Myb TAD (SBM2) is functional and mediates SUMO-binding.

In order to determine whether c-Myb binds SUMO via the putative SBMs and to identify which of the SBMs might be responsible for the non-covalent binding of SUMO, we carefully evaluated the different mutants using GST-SUMO pull-down in COS-1 lysates. When comparing c-Myb wild-type with the SBM1 mutants (L106H and A_{103}AEA) we were not able to see any difference in the ability to interact with SUMO (Fig 4*A*), and both proteins showed similar binding to SUMO-2 as the wild type. In contrast, an obvious difference was observed when comparing the SBM2 mutants (A_{267}NAA and I_{267}NII) with the wild-type. While the I_{267}NII mutant seemed to have retained the ability of c-Myb to bind SUMO, the A_{267}NAA mutant had lost this property, and only very weak background bands were detected (Fig 4*B*).

In general the same pattern emerged when using *in vitro* translated, [^{35}S]-labelled c-Myb as prey (spanning amino acid residues 89-337) in GST pull-down assays. In Fig 4*C* we show the quantified and normalized average of two independent experiments. In this system the INII mutant bound more strongly to SUMO than the wild-type, consistent with its negative influence on the activity of c-Myb (Fig. 2*A*). However, no preference for SUMO-2 was apparent and the binding of c-Myb to SUMO-1 seemed as strong as to SUMO-2. Consistent with the binding studies in COS-lysates, the ANAA mutant once again showed significantly reduced binding and was not retained on GST-SUMO more than the background seen in the GST control. We therefore conclude that c-Myb binds SUMO, with a preference for SUMO-2 under stringent conditions, through the TAD-localized SBM2, and that this interaction correlates with suppressed transactivational activity of c-Myb.

To test whether the binding of DNA to c-Myb would affect the SUMO-binding properties, we conducted some of these experiments in the presence or absence of ethidium bromide or DNA (1×GG-MRE (*mim-1A*)) [22]. However, none of these experiments indicated that the presence of Myb-responsive DNA changed the interaction between c-Myb and SUMO (data not shown). We therefore concluded that DNA does not have any influence on the non-covalent binding of SUMO to c-Myb.

AMV v-Myb still binds SUMO

Although intriguing, the mutations found in the v-Myb SBMs (L106H and I_{267}NII; c-Myb numbering) had not proved to cause any major change in the SUMO-binding properties of c-Myb (Fig 4). Still, the other oncogenic alterations in AMV v-Myb, made us consider the possibility that the SMB-mutations might have an effect in a v-Myb context. We therefore carefully compared c-Myb[1-409] and AMV v-Myb in the same experiment. As seen in Fig 5, v-Myb showed the same SUMO-binding characteristics as c-Myb, including the preferred binding to SUMO-2. From these experiments, and the mutation studies, we conclude that AMV v-Myb still binds SUMO-2 with an affinity comparable to that of c-Myb.

Mutating the SBM does not affect the sumoylation of c-Myb

Having found evidence for a physical, non-covalent interaction between c-Myb and SUMO, and observed a derepression of c-Myb activity with the SBM2 A_{267}NAA mutant that resembled the effect of the SUMO-conjugation disrupting 2KR mutations, we then asked whether their similar derepressions might simply be caused by SUMO-conjugation being dependent on a functional SBM2. The two SBM2 mutants (A_{267}NAA and I_{267}NII) were expressed in CV-1 cells and the sumoylation patterns were compared with those of c-Myb wild-type and 2KR. As can be seen in Fig 6*A*, neither the INII, nor the ANAA mutation changed the sumoylation pattern of c-Myb. This contrasts the 2KR mutant, where SUMO-conjugation is lost in both sites (K503 and K527).

Another strategy for assessing whether non-covalent SUMO-binding affects covalent SUMO-conjugation to c-Myb would be to study the synergistic properties of the SBM mutants. In a recent work we showed that c-Myb is subject to a strong synergy control, which is tightly linked to the sumoylation level of c-Myb [22]. By studying the SBM mutants in this context one might also get information relevant to identifying what mechanism is operating through this motif. We therefore carefully measured the output from our dedicated synergy reporters, only differing in the number of Myb-responsive elements. The synergy factor (SF) calculated from the relative luciferase expression from the 1×MRE and 4×MRE-containing *MYC*-reporters showed that even though the A_{267}NAA mutant had the same activity as 2KR (Fig 2*A*), its SF was well below 1.0 (Fig 6*B*). This was also the case for I_{267}NII and wild-type c-Myb, while the 2KR mutant had a SF ~ 4 (Fig 6*B*).

Altogether, these data show that mutating the SBM has no influence on the sumoylation of c-Myb. Hence, SUMO-binding and SUMO-conjugation are two independent events, controlled by separate mechanisms. Furthermore, destroying SUMO-binding has only minor effect on the synergy behaviour of c-Myb, in contrast to the large change in synergy seen after abrogating SUMO-conjugation. Thus loss of synergy control can not explain the high transactivation of the ANAA mutant.

The SBM is not controlling c-Myb activity through binding in cis to SUMO-conjugated NRD.

In the mid-nineties data were presented showing that there might exist intramolecular interactions between the EVES domain and the N-terminal region in c-Myb [32]. Moreover, others have speculated that there might be an indirect contact between the NRD and the TAD [33, 34]. With sumoylation of c-Myb being well documented [20, 21] and the present identification of a functional SBM in c-Myb TAD, we asked whether SUMO-conjugates in the NRD and the SUMO-binding motif in the start of TAD might be involved in bridging these parts of c-Myb. We reasoned that if SUMO-conjugation and SUMO-binding were part of the same mechanism in c-Myb, destroying one or both of these functions would lead to the same transactivational phenotype. As shown in Figures 2*A* and 7*A* c-Myb A_{267}NAA and c-Myb 2KR is almost identical with regard to activity, however, when both these mutations were introduced in the same construct (c-Myb ANAA 2KR) an additive increase in activity was observed (Fig 7*A*). Even when deleting the entire NRD, the A_{267}NAA mutation still increased the activity of c-Myb (Fig 7*B*). Taken together this argues against the hypothesis of a SUMO-governed bridging of the EVES and TAD domain and further supports the notion that that the transactivation potential unleashed with the SBM mutation in full-length c-Myb is disconnected from the SUMO-conjugation in the EVES domain.

Finally, we asked whether the SBM was a determinant of the strength of the neighbouring TAD. Given that c-Myb binds SUMO in TAD, elimination of this interaction by mutation might simply increase the likelihood of c-Myb interacting with the well-established co-activator p300. We therefore fused TAD wild-type and TAD ANAA to the yeast Gal4p DNA-binding domain and studied their activity in the absence or presence of p300. As can be seen in Fig 7*C* the ANAA mutation induced an increase in the transactivational activity of the isolated TAD. Moreover, both TAD wild-type and the SBM mutant seemed to respond equally well to co-expression of p300. From this we conclude that the c-Myb SUMO-binding motif is functional in a TAD-only context, and that the suppression of c-Myb activity seen with a functional SBM is a property of c-Myb that appears to be uncoupled from steric hindrance of p300.

c-Myb is binding SUMO in trans in a SBM-dependent fashion

Since our data obtained with double mutations or NRD-deletions did not support the hypothesis of an intramolecular repressive binding of SUMO, i.e. SUMO-moieties conjugated to the NRD, we assumed that the SBM had to bind SUMO in *trans*. Consequently, the repressive effect of the functional SBM had to be attributed to the recruitment of one or more sumoylated, negatively acting co-factors. If so, it should be possible to titrate out this co-repressor with an excess of free SUMO, and thereby increase Myb activity. To test this we expressed increasing amounts of non-conjugatable SUMO-1 and -2 (mono-Gly in C-terminal) in the presence of c-Myb wild-type and compared this with SUMO-binding and SUMO-conjugation negative c-Myb mutants. As can be seen in Fig 8*A*, co-expression of SUMO-1-1G in the presence of c-Myb wild-type led to an increase in c-Myb activity, while no significant change in activity was seen for c-Myb ANAA 2KR. The derepression of wild-type c-Myb was even more pronounced when titrating in increasing amounts of SUMO-2-1G (Fig 8*B*), consistent with the observed preference for SUMO-2. Interestingly, a reduced responsiveness was observed for c-Myb ANAA as well as for c-Myb 2KR, even though both proteins experienced a slight induction in transcriptional activity. The slight induction observed with c-Myb ANAA might have been caused by a weak inhibitory effect of the SUMO-1Gs on conjugation. Still there is a clear difference between the SBM mutant and wild-type c-Myb. Altogether, we conclude that c-Myb binds SUMO in *trans*. This interaction, which probably involves a negatively acting sumoylated protein, may be titrated out with increasing amounts of free SUMO, in which case SUMO-2 is the most efficient competitor. This last observation fits well with our interaction data showing that the SBM in c-Myb TAD preferably binds SUMO-2.

Co-activation of c-Myb by PML is lost when the SUMO-binding and conjugation properties of c-Myb is abrogated

Having established that c-Myb binds SUMO, most likely in the form of another sumoylated protein, we wanted to identify such a factor. Since a functional SBM in c-Myb seems to suppress c-Myb transactivation, the sumoylated factor(s) that is recruited to the c-Myb SBM probably acts as a traditional co-repressor, alternatively keeps c-Myb away from active transcription by sequestering. One such factor that might fulfil these criteria is PML. We have previously shown that PML interacts with c-Myb and recruits it to PML nuclear bodies (PML-NBs) [17]. To investigate whether the SUMO-related mutations in c-Myb influence the ability of PML to modulate c-Myb transactivation, c-Myb wild-type, ANAA, 2KR and the double mutant ANAA 2KR were transfected alone or together with PML IVa in CV-1 cells. As seen before this co-transfection led to an increase in activity for c-Myb wild-type (Fig 9; [17]). Curiously, the relative increase in activity decreased with the removal of functional SUMO-contacts; wild-type > ANAA ≈ 2KR > ANAA 2KR, where the double mutant, ANAA 2KR, was near unresponsive. We therefore conclude that PML co-activates c-Myb-dependent transcription, and that its ability to co-activate is dependent on functional SUMO-binding and SUMO-conjugation properties in c-Myb.

PML and c-Myb co-localizes in a SUMO-contact independent manner

Trying to relate the PML-induced activity alterations to PML-NB recruitment, we performed co-localization studies with PML IVa and the different c-Myb mutants. We first evaluated the distribution of the ANAA, 2KR and the ANAA 2KR when transfected

individually, and compared that to the wild-type distribution. c-Myb stains mainly uniformly in the nucleus except nucleoli, but is also found in punctual structures [17, 35]. This pattern was also seen with the ANAA, 2KR and the ANAA 2KR mutants (Fig 10*A*). Hence, abrogating c-Myb-SUMO contact does not seem to affect the general distribution of the protein within the cell. When we then looked at cells were both c-Myb and PML were ectopically expressed, the PML NBs were clearly visible with their characteristic doughnut-like, hollow spheres [36]. As shown earlier c-Myb wild-type co-localizes to these structures (Fig 10*B*; [17]). To our surprise neither loss of SUMO-binding nor SUMO-conjugation properties in c-Myb affected the recruitment to PML-NBs. Both the ANAA and the 2KR mutant, as well as the double mutant, ANAA 2KR, co-localized with PML (Fig 10*C*, *D* and *E*).

From this we conclude that c-Myb is recruited to PML nuclear bodies in a SUMO contact-independent manner, and that the lowered co-activating function of PML seen with lost c-Myb SUMO-binding and/or SUMO-conjugation is not due to altered affinity for PML-NBs.

DISCUSSION

In this work we have identified a novel SUMO-link in human c-Myb in the form of a functional SUMO-binding motif (V/I-X-V/I-V/I; V_{267}NIV) located in the N-terminal part of its transactivation domain. The functionality of this motif was assessed by two criteria: (1) c-Myb should show detectable binding affinity for SUMO, dependent on an intact SBM; and (2) removal of SBM by mutation should cause a change in the activity of c-Myb. The first criterion was assessed by GST pull-down assays with different SUMO isoforms and showed that c-Myb binds SUMO in an SBM-dependent fashion, with a clear preference for SUMO-2/3 (Fig 3). The second functionality test demonstrated that c-Myb with the SBM inactivated had become derepressed and increased its transactivation potential significantly (Fig 2).

Using the reported SUMO-binding consensus motifs [12, 13] we identified two putative SBM sites in c-Myb; one in DBD and the other in TAD. The potential SBM in the DBD was found to be positioned in the middle of the first α-helix in the second Myb-repeat (SBM1; Fig 1*A*). This was in itself a reason for rejection, since the SBMs reported so far seem to reside within short linear protein motifs found in unstructured regions [9]. Still, data on the structure of Myb R_2 in solution has been conflicting [37, 38], even though it is now generally accepted that only the third helix in R_2 is dependent on DNA-binding for its structuring [38-40]. The putative SMB in TAD (SBM2; Fig1*A*) on the other hand seemed to be in an unstructured region, based on a GlobPlot analysis. Nevertheless, since both putative SBMs turned out to be mutated in AMV v-Myb, we chose to examine both of them. Effector-reporter assays and GST pull-down with both *in vitro* translated and COS-1-expressed c-Myb showed that only mutations in the TAD-localized SBM had an effect on c-Myb activity and SUMO-binding. When destroying the potential SBMs by classical alanine substitutions (V/I/L→A) only the V_{267}NIV→A_{267}NAA mutation both derepressed c-Myb transactivational activity (Fig 2*A*) and abrogated SUMO-binding (Figs 4*BC*). We therefore conclude that only the SBM2 met our criteria for being functional. If SBM1 also contributes to SUMO-binding, it probably plays a minor role.

Recently, the determinant for SUMO isoform specificity in SBMs was shown to lie in a stretch of negatively charged residues located directly N- or C-terminally of the SBM [12]. Hecker *et al*. have demonstrated that SBMs that lack the acidic stretch tend to interact with SUMO-2/3 [12]. Since the SBM2 only consisted of a hydrophobic core (closest acidic residue: +14), we reasoned that if functional, SBM2 would be a SUMO-2/3 binding motif, and indeed this was the case: In all the different interaction assays we performed with c-Myb expressed *in vivo*, a preference for SUMO-2 binding was observed (Figs 3, 4*AB* and 5). When using *in vitro* translated protein, SUMO-1 seemed to interact as strongly with c-Myb as SUMO-2 (Fig 4*C*). Whether this difference is an effect of changes in c-Myb topology or interaction milieu; e.g. lack of factors competing for epitopes or promoting interaction, is not known. More important, when conjugated to the right factor the SUMO-moiety might bind more efficiently to c-Myb than seen in the GST pull-down assays in this study, due to additional interaction surfaces [41].

When taking a closer look at the c-Myb SBM and the nearby residues, it becomes evident that it displays a kind of symmetry; V_{267}NIVNV (Fig 1*A*). Song *et al*. has shown that SUMO is able to bind SBMs also in the reverse direction; V/I-V/I-X-V/I/L [13]. For SUMO-1 binding however, the context sequence, i.e. the localization of the acidic cluster, probably directs the orientation [12, 13]. SUMO-2/3 on the other hand, which is not dependent on these negative charges, could theoretically bind in both directions at semi-palindromic SBMs. For c-Myb and other SUMO-2/3 binding proteins with symmetric SBMs this might increase the flexibility of the interaction, and moreover, thermodynamically strengthen the binding. In this context it is interesting to notice that even though the functional significance of semi-palindromic SBMs in SUMO-2/3 binding still has not been established, such SBMs are found in many SUMO-binding factors. 12 out of 22 SBMs listed in [12] displays symmetry. This includes SBMs in Sp100, PIAS1, -2 and -3, and RanBP2. In addition the SBM in PML [9, 14], in Daxx [15], and one of the two in TDG [16] are symmetric.

The fact that both of the putative c-Myb SMBs are mutated in AMV v-Myb, motivated the initiation of this study. The finding of evolutionary conserved motifs with a designated function that is mutated in the leukemogenic v-Myb, immediately suggested a possible association. Still, only the SBM in DBD (SBM1), were mutated away from the consensus (V_{103}IELV→V_{103}IEHV). Moreover, when ruling out that SBM1 was functional (Figs 2 and 4*A*), it became clear that AMV v-Myb has not disposed it self of SUMO-binding. The I_{267}NII motif still supports binding of SUMO-2/3, both in a c-Myb (Fig. 4*BC*) and v-Myb background (Fig 5). Some of our experiments even suggested a slightly enhanced binding caused by the INII substitution (Fig 4*C*). Furthermore, v-Myb is probably still subjected to negative regulation via this motif (Figs 2*A* and 7*B*). One might ask why loss of SUMO binding is not found in v-Myb, since it would be expected to relieve it from negative control. Given the significant derepression seen with SBM2 mutated, its associated phenotype might not be selected for. Regarding mutational activation of essential gene products like c-Myb, there obviously exists a threshold limit, beyond which additional aberrations becomes lethal. Hence, subtle phenotypes may be more likely to accumulate. On the other hand, neutral mutations may occur as a natural consequence of the mutation-driven evolution. Therefore it is possible that some mutations, also in v-Myb, do not alter the phenotype.

Further evidence for a functional SUMO-binding, also in AMV v-Myb, comes from an older report using linker insertion mutagenesis in AMV v-*myb*. In an effort trying to link transactivation and transformation by v-Myb, Lane *et al*. in fact generated one insertion mutant interfering with the SBM2 element defined in this work (v-Myb 752; I_{202}NII→I_{202}NGPII) [42]. Interestingly, this mutant were able to activate transcription 25-fold more efficiently than AMV v-Myb in QT6 cells [43], suggesting that this might be due to loss of interaction with a unknown cellular inhibitor. In light of the present work, their data supports the functionality of SBM2, and consequently that v-Myb is negatively regulated by SUMO-binding.

Having identified a functional SBM in c-Myb, we sought to dissect its mechanism of action. As given for a SUMO-binding domain, we expected SBM2 to act through the binding of a sumoylated protein. Furthermore, since mutation of SBM2 caused derepression, we expected the unknown sumoylated partner to exert a repressive effect on c-Myb. Two different types of binding partners could potentially fulfil these requirements. The most plausible would be an intermolecular mechanism, where a SUMO-modified co-repressor binds to SBM2, resulting in lowered c-Myb transactivational activity. An alternative hypothesis might be that SBM2 interacts intramolecularly with sumoylated NRD, leading to a repressed conformation of c-Myb.

Before we addressed the inter- and intramolecular hypothesis, we had to exclude a rather trivial explanation, assuming that the SBM might be required for binding of SUMO as part of the c-Myb SUMO-conjugation process. Loss of SUMO-binding would then lead to reduced SUMO-conjugation and thereby increased activity. Non-covalent binding of SUMO has been indicated to be an important property in orientating SUMO for optimal conjugation [44, 45]. However, as evident from western blots of the different SMB mutants, neither the A_{267}NAA, nor the I_{267}NII mutation had any effect on the sumoylation of c-Myb (Fig 6*A*). We have

recently shown that the "synergy control (SC) phenomenon" is strongly operating on c-Myb, and that this is tightly linked to sumoylation [22]. Therefore, the observation that c-Myb ANAA still displayed SC, with a synergy factor below 1.0 (Fig 6*B*), functionally supports our conclusion that the SBM do not contribute to the sumoylation of c-Myb.

The first candidate mechanism we addressed was the intramolecular one where SUMO conjugated to the NRD of c-Myb might act as the binding partner of SBM2. Such an explanation would potentially substantiate the hypothesis of a fold-back mechanism between the EVES domain and the N-terminal region in c-Myb [32, 46] or the transactivation domain [33, 34]. Such intramolecular interactions have been hypothesized to conceal co-activator binding epitopes, thus lowering c-Myb activity. Furthermore, the transactivation domain of c-Myb was demonstrated to be activated in *trans* by c-Myb NRD when co-transfected in Gal4 tethering assays. This was in fact suggested to be caused by a universal cellular inhibitor, able bind both domains, being titrated out by the NRD [33]. The comparable activities of c-Myb 2KR and ANAA seen in our study (Figs 2*A* and 7*A*) suggested a common mechanism, given that these mutants represented two separate ways of destroying the same intramolecular bridge. However, when introducing both the 2KR and ANAA mutations in the same construct, the transcriptional activity more than doubled, resulting in an extremely active phenotype (Fig 7*A*). Moreover, the ANAA mutant still had an activating effect when the NRD, including the SUMO-modified area of c-Myb, was deleted (fig 7*B*). Finally, destroying the SUMO-binding properties of c-Myb even had an effect when studied in a TAD-only context (Fig 7*C*). Thus, these data do not fit the activity pattern anticipated if the derepression was caused by disruption of an intramolecular association between a SUMO-conjugated EVES domain and SBM2. Still these experiments do not formally exclude the possibility of a fold-back mechanism in c-Myb not involving SUMO or not leading to activity changes.

Having excluded altered SUMO-conjugation or broken intramolecular interactions as explanations to the increased transactivational potential of the SBM mutant, we addressed the possibility of intermolecular mechanisms. We reasoned that if a sumoylated, negatively acting factor was able to bind to the c-Myb SBM, it should be possible to interfere with this binding by overexpressing non-conjugatable SUMO. By co-transfecting c-Myb and increasing amounts of SUMO-1-1G and SUMO-2-1G we were able to increase the c-Myb activity, most likely by titrating out both SUMO-binding repressors (Fig 8: wt vs. ANAA) and SUMO-conjugation of c-Myb by interference with the sumoylation apparatus (Fig 8: wt vs. 2KR). This suggests that c-Myb is repressed in *trans* via SBM2, as well as via the SUMO moieties in EVES.

In principle, intermolecular mechanisms may explain our observed derepression directly or indirectly. Either, a binding partner of SBM may lead to obstruction of co-activator interaction, or it may itself act as an active co-repressor. A clear candidate for an obstructive mechanism would be interference with binding of the co-activator p300 to c-Myb TAD (binding between amino acid residues 295-309; [47-49]). However, the repressive effect seems to be uncoupled from steric hindrance of the histone acetyltransferase, since we observed that p300 co-activated TAD wild-type just as well as TAD ANAA (Fig 7*C*). Interestingly, p300 has been shown to be sumoylated, leading to the recruitment of HDAC6 and repression of p300-dependent transcription [50]. Given the close proximity of the SBM and the p300-interacting region in c-Myb it would be interesting to examine whether sumoylated p300 can bind both motifs.

One obvious candidate for an SBM-partner acting in *trans* and thus regulating c-Myb transactivation is the Promyelocytic Leukemia (PML) protein. Recently, both sumoylation and SUMO-binding have been shown to regulate nucleation of the PML protein into nuclear bodies (PML-NBs; [14, 51]). Furthermore, the sequestering of nuclear factors into the PML-NBs is also mediated through SUMO-contacts [15, 52]. As shown earlier c-Myb wild-type co-localizes with these structures [17]. When comparing this pattern (Fig 10*B*) with the co-localization of PML and the SUMO-contact mutants (ANAA, 2KR and ANAA 2KR) employing immunofluorescence, no detectable changes in localization was observed (Fig 10*C-E*). Dahle *et al*. showed that the interaction between c-Myb and PML appeared to be independent of c-Myb sumoylation, although a quantitative difference was not excluded [17]. Our new data supports this, and extend the

notion of a SUMO-independent recruitment of c-Myb to PML-NBs to include also SUMO-binding. PML and PML-NBs have been given a particular attention because of their role in human Acute Promyelocytic Leukemia (APL; [53]). Accordingly, dissecting the link between c-Myb and PML, both being implicated in leukemic disorders, is of importance.

Interestingly, and in contrast to the co-localization of c-Myb and PML, the co-activating function of PML was reduced as the SUMO-binding motif and the SUMO-acceptor lysines in c-Myb were mutated (Fig 9). We believe that this co-activation might be caused by PML-NBs sequestering negatively acting co-factors acting on c-Myb through the SBM or conjugated SUMO moieties, and furthermore, that the reduced PML co-activation seen with the SUMO-contact mutants is due to co-repressor interactions already being broken. Indeed, PML has been shown to function as a co-activator for the glucocorticoid receptor through sequestering the SUMO-binding co-repressor Daxx to PML-NBs [15, 54]. In their work on SUMO-2/3 conjugation of c-Myb Sramko *et al*. observed a derepression of c-Myb activity using tricostatin A [21]. This indicates that we might be looking at an HDAC being recruited to the SUMO conjugates. Concerning what negative-acting factor(s) that binds to the c-Myb SBM, a follow-up study of the linker insertion mutagenesis hitting the SBM in v-Myb might give some directions. When the v-Myb I_{202}GPNII mutant was tested in yeast cells, it only induced transcription about two-fold more than v-Myb, as compared to 25-fold more than v-Myb in quail fibroblasts [43]. This narrows the candidates down to vertebrate-specific proteins. Still, it might just indicate that the yeast SUMO homologue, Smt3p, is unable to interact with the c-Myb SBM. However, the affinity of Smt3p for the SBM in PIAS2 (V_{467}DVIDL) is similar to that of human SUMO [12, 13]

In conclusion, we have identified a site in c-Myb being important for the ability of the factor to associate with SUMO. The mutation of this motif causes a significant derepression of the activity of c-Myb. Our observations point in the direction of an intermolecular rather than an intramolecular mechanism. However, the identity of the assumed SBM-binding repressor remains to be determined. Therefore, identifying SUMO-contact dependent co-repressors acting on c-Myb is an important task for future work.

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REFERENCES

1. Oh, I.H. & Reddy, E.P. (1999) The myb gene family in cell growth, differentiation and apoptosis. *Oncogene* **18**, 3017-33.
2. Gill, G. (2004) SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev* **18**, 2046-59.
3. Gill, G. (2005) Something about SUMO inhibits transcription. *Curr Opin Genet Dev* **15**, 536-41.
4. Hay, R.T. (2005) SUMO: a history of modification. *Mol Cell* **18**, 1-12.
5. Kerscher, O., Felberbaum, R. & Hochstrasser, M. (2006) Modification of Proteins by Ubiquitin and Ubiquitin-Like Proteins. *Annu Rev Cell Dev Biol*.
6. Hietakangas, V., Anckar, J., Blomster, H.A., Fujimoto, M., Palvimo, J.J., Nakai, A. & Sistonen, L. (2006) PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc Natl Acad Sci U S A* **103**, 45-50.
7. Yang, S.H., Galanis, A., Witty, J. & Sharrocks, A.D. (2006) An extended consensus motif enhances the specificity of substrate modification by SUMO. *EMBO J* **25**, 5083-93.
8. Saitoh, H. & Hinchey, J. (2000) Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J Biol Chem* **275**, 6252-8.
9. Song, J., Durrin, L.K., Wilkinson, T.A., Krontiris, T.G. & Chen, Y. (2004) Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *Proc Natl Acad Sci U S A* **101**, 14373-8.
10. Minty, A., Dumont, X., Kaghad, M. & Caput, D. (2000) Covalent Modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. *J Biol Chem* **275**, 36316-36323.
11. Hannich, J.T., Lewis, A., Kroetz, M.B., Li, S.J., Heide, H., Emili, A. & Hochstrasser, M. (2005) Defining the SUMO-modified proteome by multiple approaches in Saccharomyces cerevisiae. *J Biol Chem* **280**, 4102-10.
12. Hecker, C.M., Rabiller, M., Haglund, K., Bayer, P. & Dikic, I. (2006) Specification of SUMO1- and SUMO2-interacting motifs. *J Biol Chem* **281**, 16117-27.
13. Song, J., Zhang, Z., Hu, W. & Chen, Y. (2005) Small ubiquitin-like modifier (SUMO) recognition of a SUMO binding motif: a reversal of the bound orientation. *J Biol Chem* **280**, 40122-9.
14. Shen, T.H., Lin, H.K., Scaglioni, P.P., Yung, T.M. & Pandolfi, P.P. (2006) The mechanisms of PML-nuclear body formation. *Mol Cell* **24**, 331-9.

15. Lin, D.Y. et al. (2006) Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Mol Cell* **24**, 341-54.
16. Mohan, R.D., Rao, A., Gagliardi, J. & Tini, M. (2007) SUMO-1-dependent allosteric regulation of thymine DNA glycosylase alters subnuclear localization and CBP/p300 recruitment. *Mol Cell Biol* **27**, 229-43.
17. Dahle, O., Bakke, O. & Gabrielsen, O.S. (2004) c-Myb associates with PML in nuclear bodies in hematopoietic cells. *Exp Cell Res* **297**, 118-26.
18. Ramsay, R.G. (2005) c-Myb a stem-progenitor cell regulator in multiple tissue compartments. *Growth Factors* **23**, 253-61.
19. Bies, J., Markus, J. & Wolff, L. (2002) Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity. *J Biol Chem* **277**, 8999-9009.
20. Dahle, O., Andersen, T.O., Nordgard, O., Matre, V., Del Sal, G. & Gabrielsen, O.S. (2003) Transactivation properties of c-Myb are critically dependent on two SUMO-1 acceptor sites that are conjugated in a PIASy enhanced manner. *Eur J Biochem* **270**, 1338-48.
21. Sramko, M., Markus, J., Kabat, J., Wolff, L. & Bies, J. (2006) Stress-induced inactivation of the c-Myb transcription factor through conjugation of SUMO-2/3 proteins. *J Biol Chem* **281**, 40065-75.
22. Molvaersmyr, A.K., Saether, T., Lorenzo, P., Kvaloey, H., Matre, V. & Gabrielsen, O.S. (2007) SUMO-conjugation of the transcription factor c-Myb controls cooperative behaviour and induces an activator-to-repressor switch in the negative regulatory domain. *Manuscript*.
23. Saether, T., Berge, T., Ledsaak, M., Matre, V., Alm-Kristiansen, A.H., Dahle, O., Aubry, F. & Gabrielsen, O.S. (2007) The chromatin remodeling factor Mi-2alpha acts as a novel co-activator for human c-Myb. *J Biol Chem* **282**, 13994-4005.
24. Andersson, K.B., Kowenz-Leutz, E., Brendeford, E.M., Tygsett, A.H., Leutz, A. & Gabrielsen, O.S. (2003) Phosphorylation-dependent Down-regulation of c-Myb DNA Binding Is Abrogated by a Point Mutation in the v-myb Oncogene. *J Biol Chem* **278**, 3816-24.
25. Matre, V., Hovring, P.I., Fjeldheim, A.K., Helgeland, L., Orvain, C., Andersson, K.B., Gautvik, K.M. & Gabrielsen, O.S. (2003) The human neuroendocrine thyrotropin-releasing hormone receptor promoter is activated by the haematopoietic transcription factor c-Myb. *Biochem J* **372**, 851-9.
26. Eckner, R., Ewen, M.E., Newsome, D., Gerdes, M., DeCaprio, J.A., Lawrence, J.B. & Livingston, D.M. (1994) Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev* **8**, 869-84.
27. Fogal, V., Gostissa, M., Sandy, P., Zacchi, P., Sternsdorf, T., Jensen, K., Pandolfi, P.P., Will, H., Schneider, C. & Del Sal, G. (2000) Regulation of p53 activity in nuclear bodies by a specific PML isoform. *EMBO J* **19**, 6185-95.
28. Kadoya, T., Yamamoto, H., Suzuki, T., Yukita, A., Fukui, A., Michiue, T., Asahara, T., Tanaka, K., Asashima, M. & Kikuchi, A. (2002) Desumoylation activity of Axam, a novel Axin-binding protein, is involved in downregulation of beta-catenin. *Mol Cell Biol* **22**, 3803-19.
29. Tatham, M.H., Jaffray, E., Vaughan, O.A., Desterro, J.M., Botting, C.H., Naismith, J.H. & Hay, R.T. (2001) Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem* **276**, 35368-74.
30. Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F. & Grosschedl, R. (2001) PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. *Genes Dev* **15**, 3088-103.
31. Gabrielsen, O.S., Sentenac, A. & Fromageot, P. (1991) Specific DNA binding by c-Myb: evidence for a double helix-turn-helix- related motif. *Science* **253**, 1140-3.
32. Dash, A.B., Orrico, F.C. & Ness, S.A. (1996) The EVES motif mediates both intermolecular and intramolecular regulation of c-Myb. *Genes Dev* **10**, 1858-69.
33. Vorbrueggen, G., Kalkbrenner, F., Guehmann, S. & Moelling, K. (1994) The carboxyterminus of human c-myb protein stimulates activated transcription in trans. *Nucleic Acids Res* **22**, 2466-75.
34. Dubendorff, J.W., Whittaker, L.J., Eltman, J.T. & Lipsick, J.S. (1992) Carboxy-terminal elements of c-Myb negatively regulate transcriptional activation in cis and in trans. *Genes Dev* **6**, 2524-35.
35. Alm-Kristiansen, A.H., Saether, T., Matre, V., Gilfillan, S., Dahle, O. & Gabrielsen, O.S. (2007) FLASH acts as a co-activator of the transcription factor c-Myb and localizes to active RNA polymerase II foci. *Submitted to Oncogene*.
36. Janderova-Rossmeislova, L., Novakova, Z., Vlasakova, J., Philimonenko, V., Hozak, P. & Hodny, Z. (2007) PML protein association with specific nucleolar structures differs in normal, tumor and senescent human cells. *J Struct Biol* **159**, 56-70.
37. Ogata, K., Morikawa, S., Nakamura, H., Hojo, H., Yoshimura, S., Zhang, R., Aimoto, S., Ametani, Y., Hirata, Z. & Sarai, A. (1995) Comparison of the free and DNA-complexed forms of the DNA-binding domain from c-Myb. *Nat Struct Biol* **2**, 309-20.
38. Carr, M.D., Wollborn, U., McIntosh, P.B., Frenkiel, T.A., McCormick, J.E., Bauer, C.J., Klempnauer, K.H. & Feeney, J. (1996) Structure of the B-Myb DNA-binding domain in solution and evidence for multiple conformations in the region of repeat-2 involved in DNA binding: implications for sequence-specific DNA binding by Myb proteins. *Eur J Biochem* **235**, 721-35.
39. Jamin, N., Gabrielsen, O.S., Gilles, N., Lirsac, P.N. & Toma, F. (1993) Secondary structure of the DNA-binding domain of the c-Myb oncoprotein in solution. A multidimensional double and triple heteronuclear NMR study. *Eur J Biochem* **216**, 147-54.
40. Myrset, A.H., Bostad, A., Jamin, N., Lirsac, P.N., Toma, F. & Gabrielsen, O.S. (1993) DNA and redox state induced conformational changes in the DNA-binding domain of the Myb oncoprotein. *EMBO J* **12**, 4625-33.
41. Geiss-Friedlander, R. & Melchior, F. (2007) Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol* **8**, 947-56.

42. Lane, T., Ibanez, C., Garcia, A., Graf, T. & Lipsick, J. (1990) Transformation by v-myb correlates with trans-activation of gene expression. *Mol Cell Biol* **10**, 2591-8.
43. Chen, R.H. & Lipsick, J.S. (1993) Differential transcriptional activation by v-myb and c-myb in animal cells and Saccharomyces cerevisiae. *Mol Cell Biol* **13**, 4423-31.
44. Reverter, D. & Lima, C.D. (2005) Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. *Nature* **435**, 687-92.
45. Tatham, M.H., Kim, S., Jaffray, E., Song, J., Chen, Y. & Hay, R.T. (2005) Unique binding interactions among Ubc9, SUMO and RanBP2 reveal a mechanism for SUMO paralog selection. *Nat Struct Mol Biol* **12**, 67-74.
46. Karafiat, V., Dvorakova, M., Pajer, P., Kralova, J., Horejsi, Z., Cermak, V., Bartunek, P., Zenke, M. & Dvorak, M. (2001) The leucine zipper region of Myb oncoprotein regulates the commitment of hematopoietic progenitors. *Blood* **98**, 3668-76.
47. Dai, P., Akimaru, H., Tanaka, Y., Hou, D.X., Yasukawa, T., Kanei-Ishii, C., Takahashi, T. & Ishii, S. (1996) CBP as a transcriptional coactivator of c-Myb. *Genes Dev* **10**, 528-40.
48. Oelgeschlager, M., Janknecht, R., Krieg, J., Schreek, S. & Luscher, B. (1996) Interaction of the co-activator CBP with Myb proteins: effects on Myb-specific transactivation and on the cooperativity with NF-M. *Embo J* **15**, 2771-80.
49. Zor, T., De Guzman, R.N., Dyson, H.J. & Wright, P.E. (2004) Solution structure of the KIX domain of CBP bound to the transactivation domain of c-Myb. *J Mol Biol* **337**, 521-34.
50. Girdwood, D., Bumpass, D., Vaughan, O.A., Thain, A., Anderson, L.A., Snowden, A.W., Garcia-Wilson, E., Perkins, N.D. & Hay, R.T. (2003) P300 transcriptional repression is mediated by SUMO modification. *Mol Cell* **11**, 1043-54.
51. Zhong, S., Muller, S., Ronchetti, S., Freemont, P.S., Dejean, A. & Pandolfi, P.P. (2000) Role of SUMO-1-modified PML in nuclear body formation. *Blood* **95**, 2748-52.
52. Best, J.L., Ganiatsas, S., Agarwal, S., Changou, A., Salomoni, P., Shirihai, O., Meluh, P.B., Pandolfi, P.P. & Zon, L.I. (2002) SUMO-1 protease-1 regulates gene transcription through PML. *Mol Cell* **10**, 843-55.
53. Salomoni, P. & Pandolfi, P.P. (2002) The role of PML in tumor suppression. *Cell* **108**, 165-70.
54. Lin, D.Y., Lai, M.Z., Ann, D.K. & Shih, H.M. (2003) Promyelocytic leukemia protein (PML) functions as a glucocorticoid receptor co-activator by sequestering Daxx to the PML oncogenic domains (PODs) to enhance its transactivation potential. *J Biol Chem* **278**, 15958-65.

FIGURE LEGENDS

Figure 1 c-Myb contains two putative SUMO-binding motifs which are both mutated in AMV v-Myb. *A*, Schematic presentation of human c-Myb with its two potential SUMO-binding motifs, one residing in the R2 repeat of DBD and the other in the N-terminal part of TAD. DBD: DNA-binding domain, TAD: transactivation domain, NRD: negative regulatory domain, R1, -2, -3: Myb repeat 1, 2 and 3, TP/CR: Thr- and Pro-rich conserved region, FAETL and EVES: motifs found within the assigned regions, LZ: putative leucine zipper. *B*, Multiple sequence alignment of the areas harbouring the SUMO-binding motifs (boxed), using different mammalian c-Mybs. The acidic streches close to the SBMs are indicated by a solid line. AMV v-Myb is included for comparison. The consensus SUMO-binding motifs suggested by Song *et al*. 2005 and Hecker *et al*. 2006 are included for clarity. *C,* The SBM mutants used in this work. No change in amino acid residue is marked with "-".

Figure 2 Mutating the SBM in c-Myb TAD derepress c-Myb and makes it super active.
A, CV-1 cells were transfected with a Myb-responsive *3×MRE(GG)-MYC* reporter plasmid (0.2 μg) and plasmids encoding full-length c-Myb wt, 2KR, AAEA, L106H, ANAA or INII in increasing amounts (0.2-0.4 μg). The results are presented as relative luciferase units (RLU). The results represent the mean RLU±SEM of at least three independent assays performed in triplicates. *B*, CV-1 cells were transfected with plasmids encoding c-Myb-HA wt, 2KR, AAEA, L106H, ANAA or INII (0.2 μg). Cell lysates were subjected to SDS-PAGE and immunoblot analysis was performed using an anti-HA antibody.

Figure 3 Human c-Myb binds SUMO. *In vitro* binding assays were performed in lysates from COS-1 cells transfected with *A*, pCIneoB-3FLAG-hcM[1-409] or *B*, pCIneoB-3FLAG-hcM[1-409] and pCIneoB-3FLAG-hcM. The lysate was incubated with comparable amounts of GST-SUMO-1 and -SUMO-2 fusion proteins. The bound proteins were analyzed by SDS-PAGE and immunoblot analysis using an anti-FLAG antibody. 5 % of the input (total cell extract) used for the pull-down was loaded as reference. The amount of GST and GST fusion proteins was evaluated with Ponceau S red staining of the membrane after immunoblotting.

Figure 4 Only the putative SBM in c-Myb TAD (SBM2) is functional and mediates SUMO-binding. *In vitro* binding assays were performed in lysates from COS-1 cells transfected with *A*, pCIneoB-3FLAG-hcM[1-409] wt and the SBM1 mutants AAEA and L106H and *B*, pCIneoB-3FLAG-hcM[1-409] wt and the SBM2 mutants ANAA and INII. The lysates were incubated with comparable amounts of GST-SUMO-1 and SUMO-2 fusion proteins. The bound proteins were analyzed by SDS-PAGE and immunoblot analysis was performed using an anti-FLAG antibody. 5 % of the input (total cell extract) used for the pull-down was loaded as reference. The amount of GST and GST fusion proteins was evaluated with Ponceau S red staining of the membrane after immunoblotting. *C*, *In vitro* binding assays were performed with *in vitro* translated, [^{35}S]-Methionine labeled human c-Myb R2-TAD (amino acid residues 89-337) wt and the SBM2 mutants ANAA and INII. The proteins were incubated with comparable amounts of GST-SUMO-1 and SUMO-2 fusion proteins. The bound proteins were analyzed by SDS-PAGE and the amount of GST and GST fusion proteins was evaluated with Comassie Brilliant Blue staining of the gel before the [^{35}S]-signals were read with a Typhon phoshoimager (GE Healthcare). The signals from the input controls (loaded as reference) were used to normalize the data acquired with ImageQuant TL v2003.02 (GE Healthcare). The results represent the mean % retention of the total *in vitro* translated protein ± SD of two independent assays.

Figure 5 AMV v-Myb still binds SUMO. *In vitro* binding assays were performed in lysates from COS-1 cells transfected with pCIneoB-3FLAG-hcM[1-409] wt and pCIneoB-3FLAG-AMV. The lysates were incubated with comparable amounts of GST-SUMO-1 and SUMO-2 fusion proteins. The bound proteins were analyzed by SDS-PAGE and immunoblot analysis using an anti-FLAG antibody. 5 % of the input (total cell extract) used for the pull-down was loaded as reference. The

amount of GST and GST fusion proteins was evaluated with Ponceau S red staining of the membrane after immunoblotting.

Figure 6 Mutating the SBM does not affect the sumoylation of c-Myb. *A*, CV-1 cells were transfected with plasmids encoding c-Myb-HA wt, ANAA, INII or 2KR (1.0 μg) in combination with a small input of PIASy expression plasmid (0.25 μg). Cells were scraped in cold PBS and lysed directly by sonication in SDS loading-buffer. The lysates were subjected to SDS-PAGE and immunoblot analysis using an anti-HA antibody. *B*, CV-1 cells were transfected with the c-Myb responsive *1×MRE(GG)-MYC* or *4×MRE(GG)-MYC* reporter plasmids (0.2 μg) and plasmids encoding full-length c-Myb wt, ANAA, INII or 2KR (0.2 μg) The results are presented as Synergy Factor (SF) calculated from the relative luciferase units ($SF=RLU_{4\times MRE}/(4\times RLU_{1\times MRE})$ [22]. The results represent the mean SF±SEM of at least three independent assays performed in triplicates.

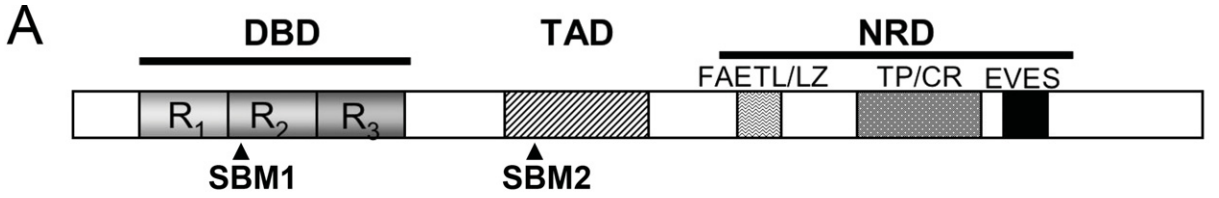
Figure 7 The SBM is not controlling c-Myb activity through binding in *cis* to SUMO-conjugated NRD. *A*, CV-1 cells were transfected with a Myb-responsive *3×MRE(GG)-MYC* reporter plasmid (0.2 μg) and plasmids encoding full-length c-Myb wt, 2KR, ANAA or ANAA 2KR (0.4 μg). *B*, CV-1 cells were transfected with a Myb-responsive *3×MRE(GG)-MYC* reporter plasmid (0.2 μg) and plasmids encoding c-Myb[1-409] wt, AAEA, L106H, ANAA or INII in increasing amounts (0.2-0.4 μg). *C*, CV-1 cells were transfected with 2.5 ng (grey bars) or 5.0 ng (black bars) of a plasmid expressing Gal4p-DBD fused to c-Myb TAD wt or ANAA (amino acid residues 259-337) in the absence or presence (0.1 μg) of a p300 expression plasmid. The reporter output from the *SNRPN*-driven Gal4p-responsive reporter plasmid (0.2 μg) was normalized to the effect of Gal4p-DBD, which was set to 100. The results are presented as relative luciferase units (RLU). The results represent the mean RLU±SEM of at least three independent assays performed in triplicates.

Figure 8 c-Myb is binding SUMO in *trans* in a SBM-dependent fashion. CV-1 cells were transfected with a Myb-responsive *3×MRE(GG)-MYC* reporter plasmid (0.2 μg) and plasmids encoding full-length c-Myb wild-type, ANAA, 2KR or ANAA 2KR (0.2 μg), in combination with increasing amounts (0-0.4 μg) of *A*, SUMO-1-1G or *B*, SUMO-2-1G (conjugation-deficient mutants). Increasing amounts of SUMO-1-1G or SUMO-2-1G (0–0.4 μg) were also transfected singularly together with the reporter. The results are presented as fold-induction of relative luciferase units (RLU), and the activities of the different c-Myb proteins in the absence of SUMO is set to 1.0. The results represent the mean RLU±SEM of at least three independent assays performed in triplicates.

Figure 9 Co-activation of c-Myb by PML is lost when the SUMO-binding and conjugation properties of c-Myb is abrogated. CV-1 cells were transfected with a Myb-responsive *3×MRE(GG)-MYC* reporter plasmid (0.2 μg) and plasmids encoding full-length c-Myb wt, 2KR, ANAA or ANAA 2KR (0.2 μg), alone or together with PML IVa (0.2 μg). The results are presented as fold-induction of relative luciferase units (RLU), and the activities of the different c-Myb proteins in the absence of PML is set to 1.0. The results represent the mean RLU±SEM of at least three independent assays performed in triplicates.

Figure 10 PML and c-Myb co-localizes in a SUMO-contact independent manner. *A,* CV-1 cells were transfected with plasmids encoding HA-tagged c-Myb wild-type, c-Myb ANAA, c-Myb 2KR or c-Myb ANAA 2KR (0.3 μg) as indicated in the figure and analyzed by indirect immunofluorescence and confocal microscopy. The c-Myb proteins were detected with rabbit anti-HA antibody and Alexa Fluor 488 goat anti-rabbit IgG(H+L). *B-E,* CV-1 cells were transfected with plasmids encoding PML IVa (0.15 μg) and HA-tagged c-Myb wild type (*B*), c-Myb ANAA (*C*), c-Myb 2KR (*D*) or c-Myb ANAA 2KR (*E*) (0.3 μg) and analyzed as above. The c-Myb proteins were detected as in A (green signal) and PML was detected with mouse anti-PML antibody and Alexa Fluor 633 goat anti-mouse IgG1 (red signal). Co-localization is visualized as yellow colour when merging the images (right panel). DNA was labelled by Hoechst 33258 (blue signal).

FIGURE 1



B

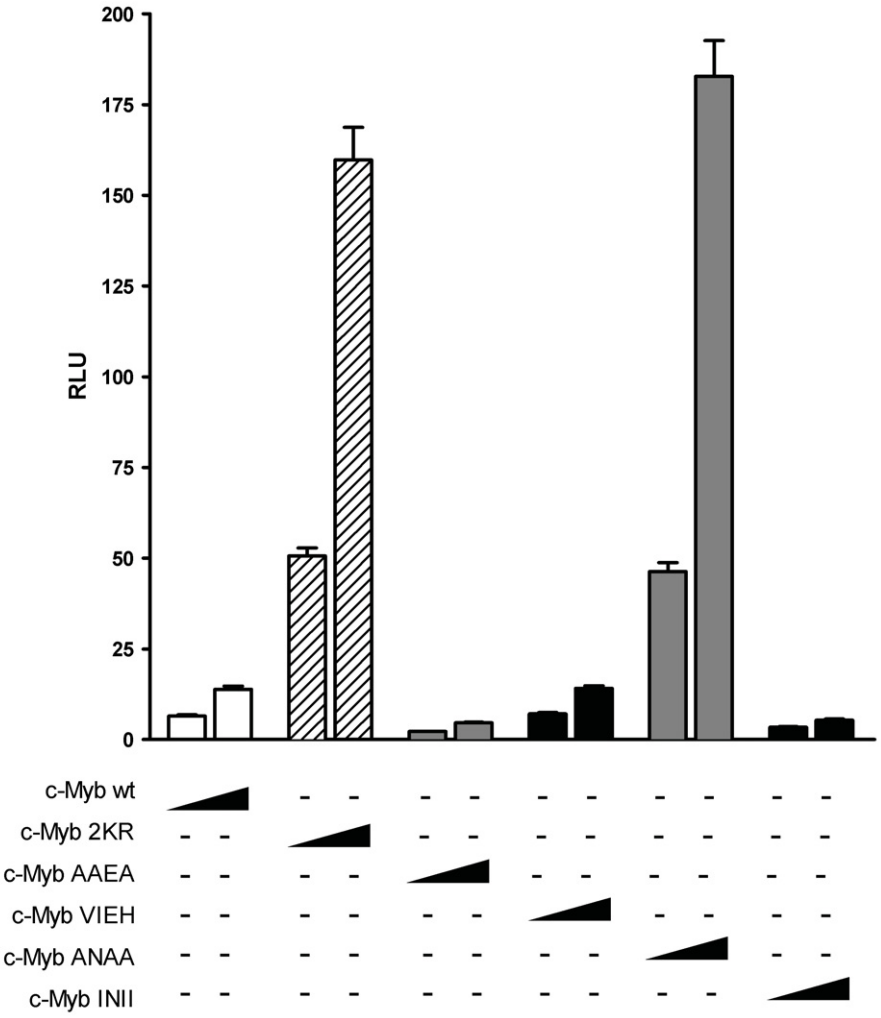
	95 SBM1 109	265 SBM2 292
c-Myb human	WTKEEDQR VIELV QK	LH VNIVNV PQPAAAAIQRHYNDEDPEKE
c-Myb bovine	WTKEEDQR VIELV QK	LH VNIVNV PQPAAAAIQRHYNDEDPEKE
c-Myb murine	WTKEEDQR VIELV QK	LH VNIVNV PQPAAAAIQRHYNDEDPEKE
c-Myb rattus	WTKEEDQR VIELV QK	LH VNIVNV PQPAAAAIQRHYNDEDPEKE
c-Myb chicken	WTKEEDQR VIELV QK	LH VNIVNV PQPAAAAIQRHYNDEDPEKE
c-Myb xenopus	WTKEEDQR VIELV HK	LR VNIVNV PQLATAPVQRHYNDEDPEKE
c-Myb oryzias	WTKEEDQR VIELV QK	LR FNILNI PQHGTAAIQRHYGEEDPEKE
	******** ***** :*	*: .**:*: ** .:*.:****.:******
v-Myb AMV	WTKEEDQR VIEHV QK	LH INIINV PQPAAAAIQRHYTDEDPEKE
Consensus:	aaa-[V/I][V/I]X[V/I/L]	[V/I]X[V/I][V/I]-aaa

C

c-Myb AAEA	AA-A-	------
c-Myb L106H	---H-	------
c-Myb ANAA	-----	A-AA--
c-Myb INII	-----	I-II--

FIGURE 2

A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150
125
100
75
50
25
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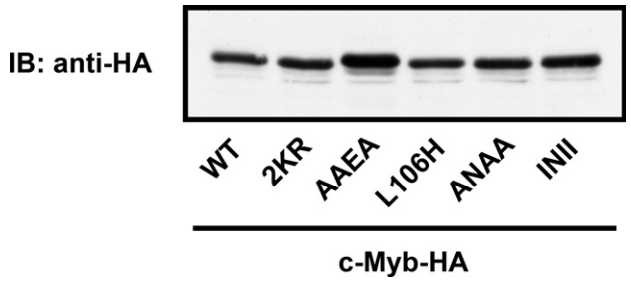
c-Myb ANAA
c-Myb INII

B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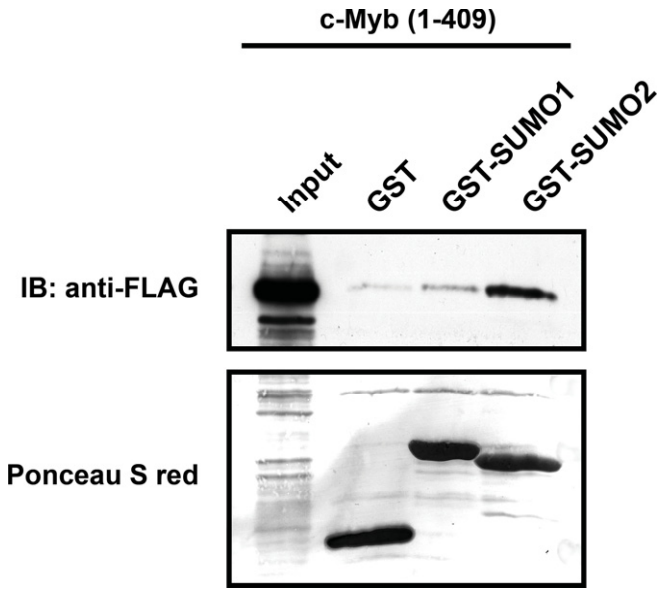
WT
2KR
AAEA
L106H
ANAA
INII
c-Myb-HA

FIGURE 3

A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GST-SUMO2
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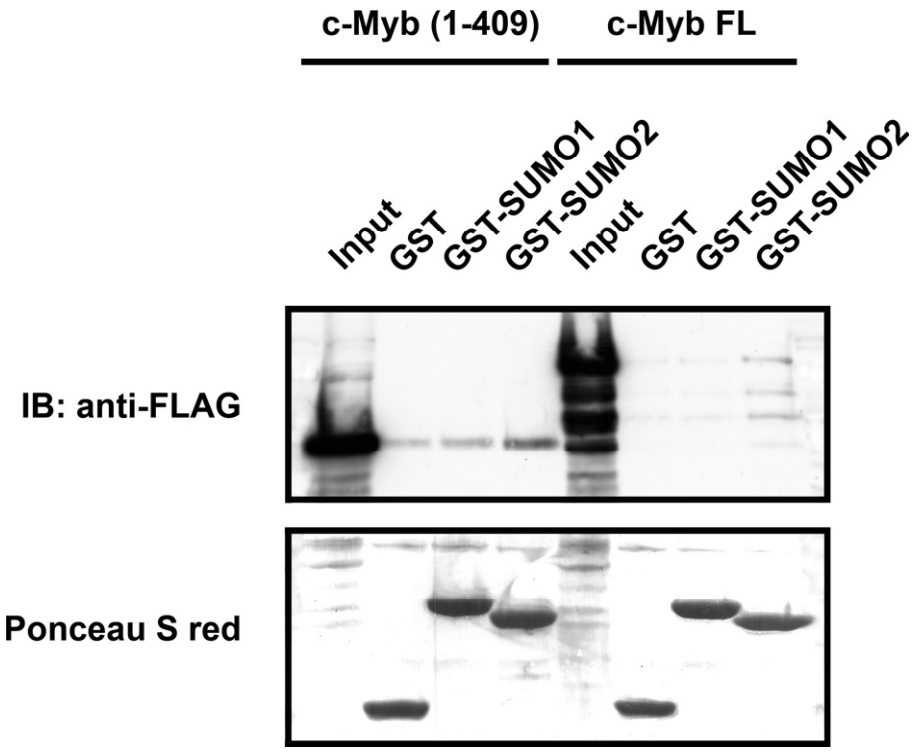
Ponceau S red

B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c-Myb FL
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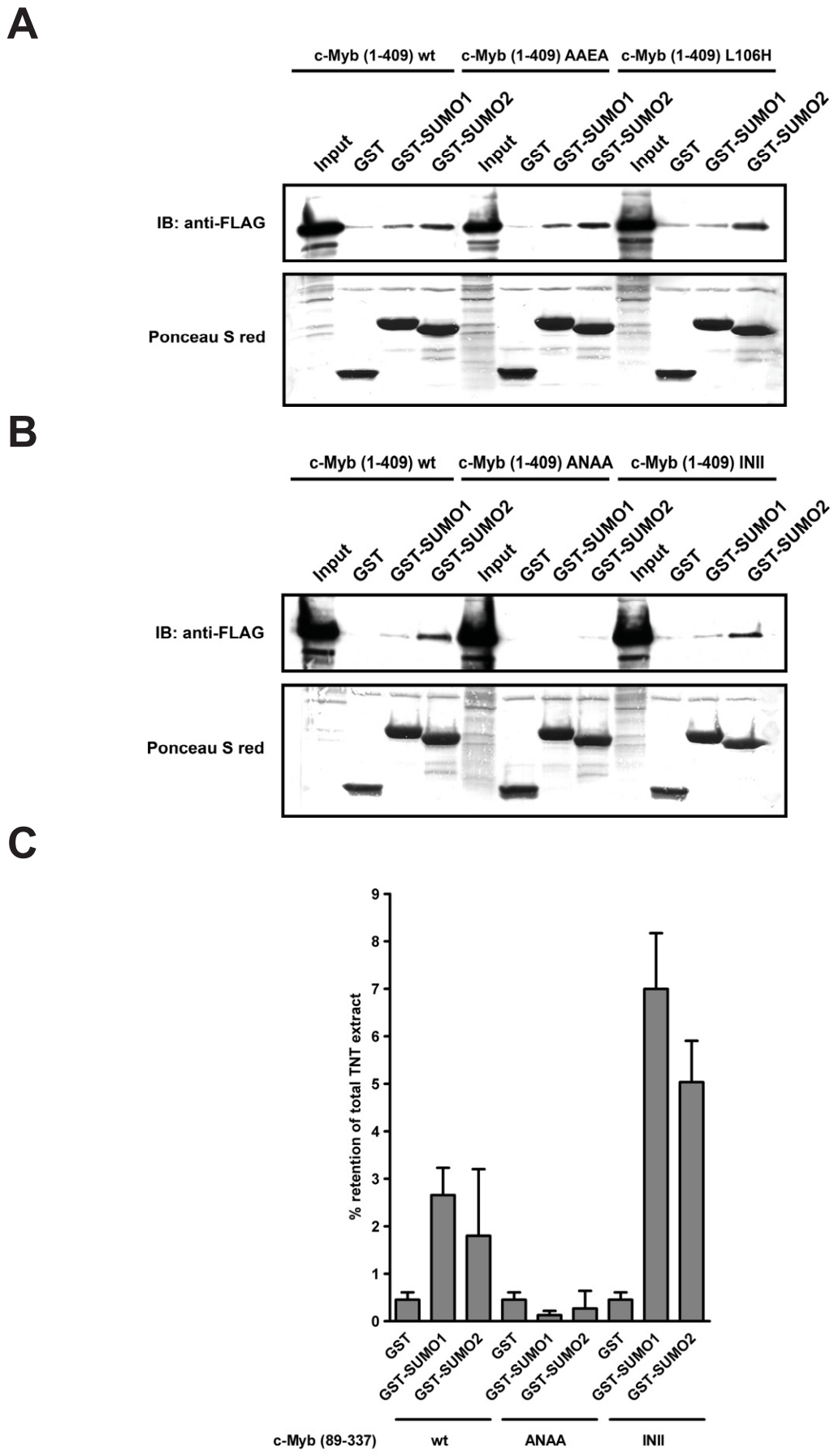
GST-SUMO2
Input
GST
GST-SUMO1
GST-SUMO2
IB: anti-FLAG
Ponceau S red

FIGURE 4


A
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c-Myb (1-409) AAEA
c-Myb (1-409) L106H
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GST-SUMO2
IB: anti-FLAG
Ponceau S red
B
c-Myb (1-409) wt
c-Myb (1-409) ANAA
c-Myb (1-409) INII
Input
GST
GST-SUMO1
GST-SUMO2
IB: anti-FLAG
Ponceau S red
C
% retention of total TNT extract
0
1
2
3
4
5
6
7
8
9
GST
GST-SUMO1
GST-SUMO2
c-Myb (89-337)
wt
ANAA
INII

FIGURE 5

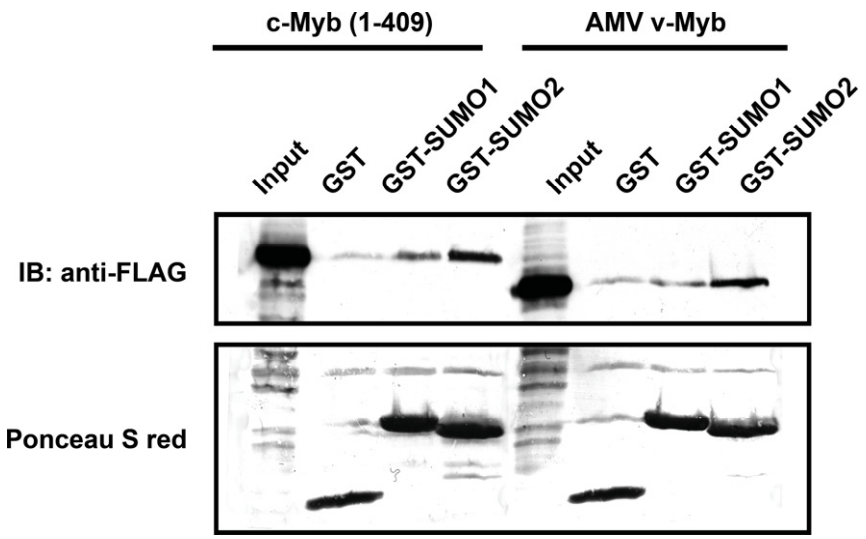
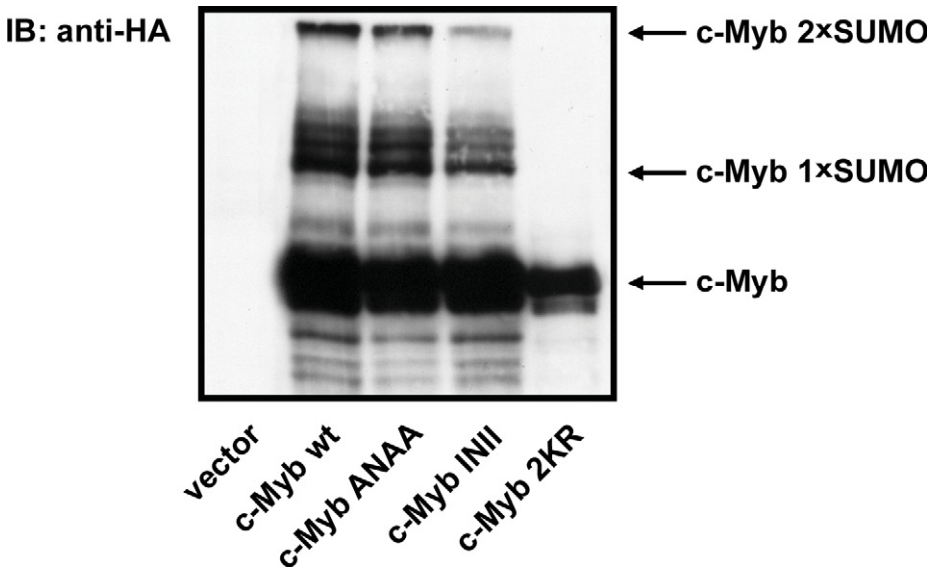

c-Myb (1-409)
AMV v-Myb
Input
GST
GST-SUMO1
GST-SUMO2
Input
GST
GST-SUMO1
GST-SUMO2
IB: anti-FLAG
Ponceau S red

FIGURE 6

A


IB: anti-HA
c-Myb 2×SUMO
c-Myb 1×SUMO
c-Myb
vector
c-Myb wt
c-Myb ANAA
c-Myb INII
c-Myb 2KR

B

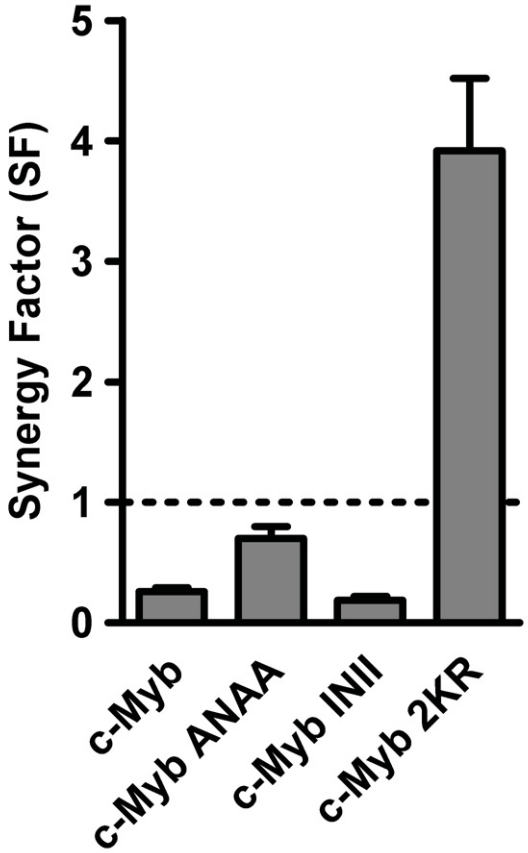
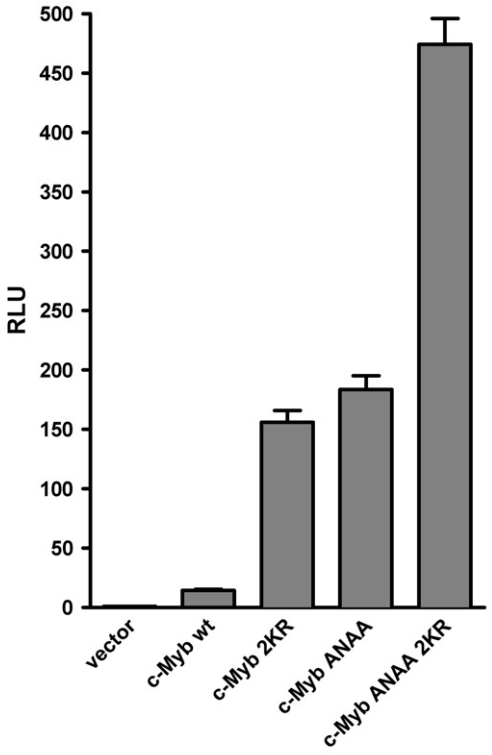

Synergy Factor (SF)
5
4
3
2
1
0
c-Myb
c-Myb ANAA
c-Myb INII
c-Myb 2KR

FIGURE 7

A


500
450
400
350
300
250
200
150
100
50
0
RLU
vector
c-Myb wt
c-Myb 2KR
c-Myb ANAA
c-Myb ANAA 2KR

B

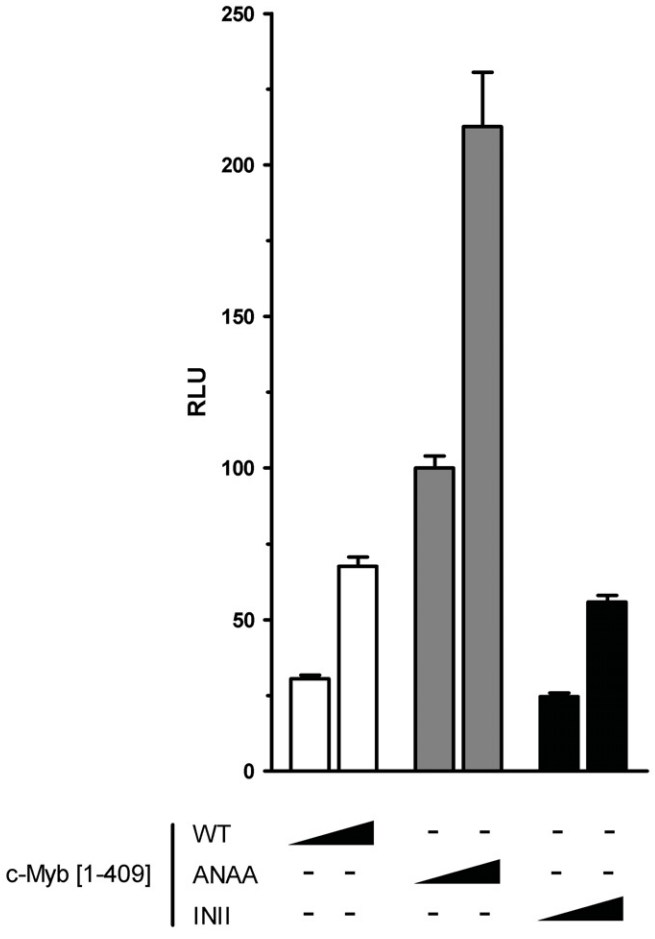

250
200
150
100
50
0
RLU
c-Myb [1-409]
WT
ANAA
INII

FIGURE 7

C

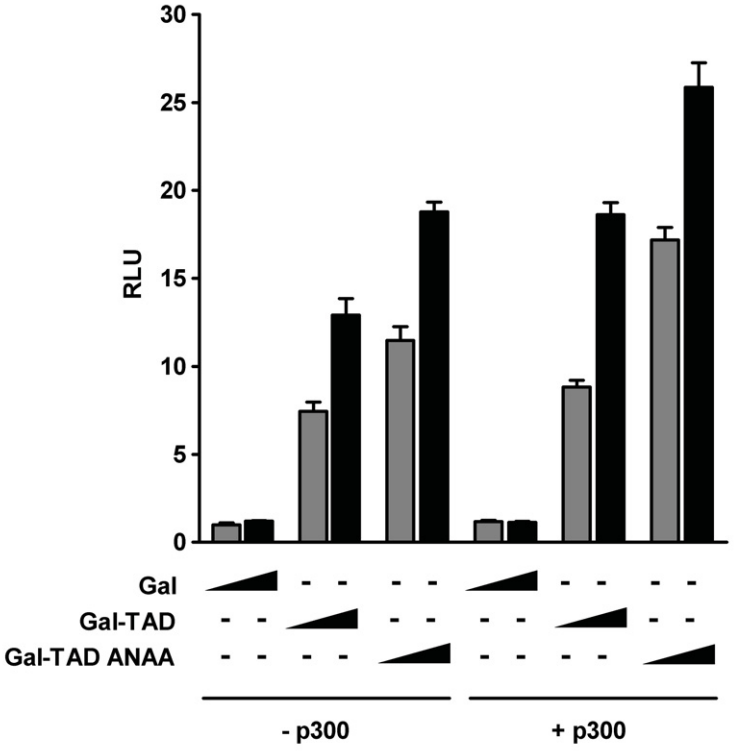
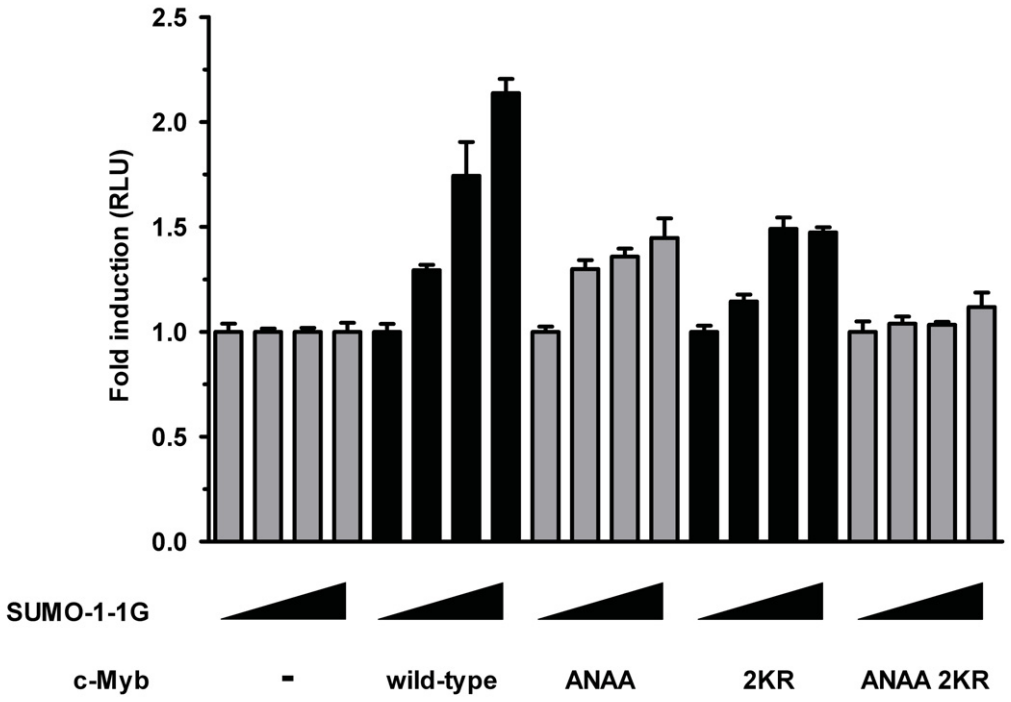


FIGURE 8

A


2.5
2.0
1.5
1.0
0.5
0.0
Fold induction (RLU)
SUMO-1-1G
c-Myb
-
wild-type
ANAA
2KR
ANAA 2KR

B

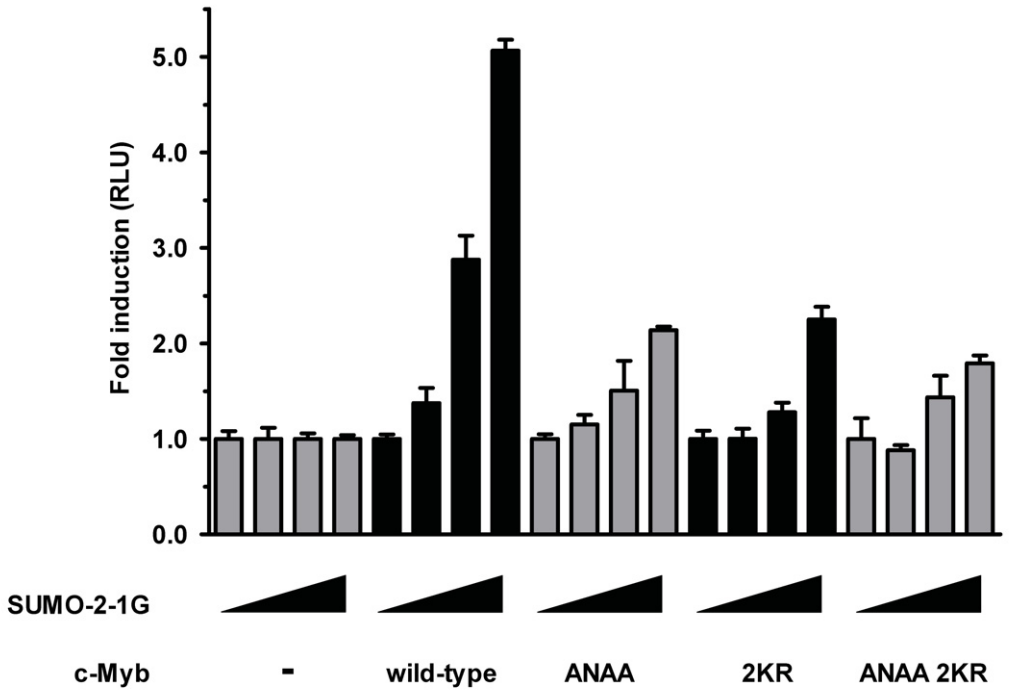

5.0
4.0
3.0
2.0
1.0
0.0
Fold induction (RLU)
SUMO-2-1G
c-Myb
-
wild-type
ANAA
2KR
ANAA 2KR

FIGURE 9

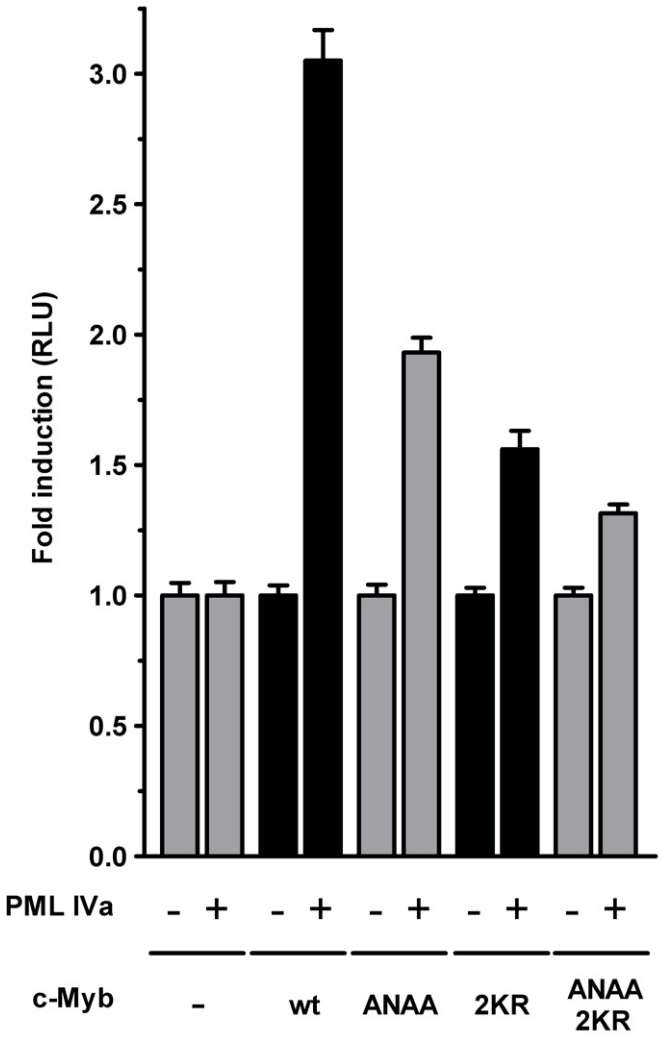
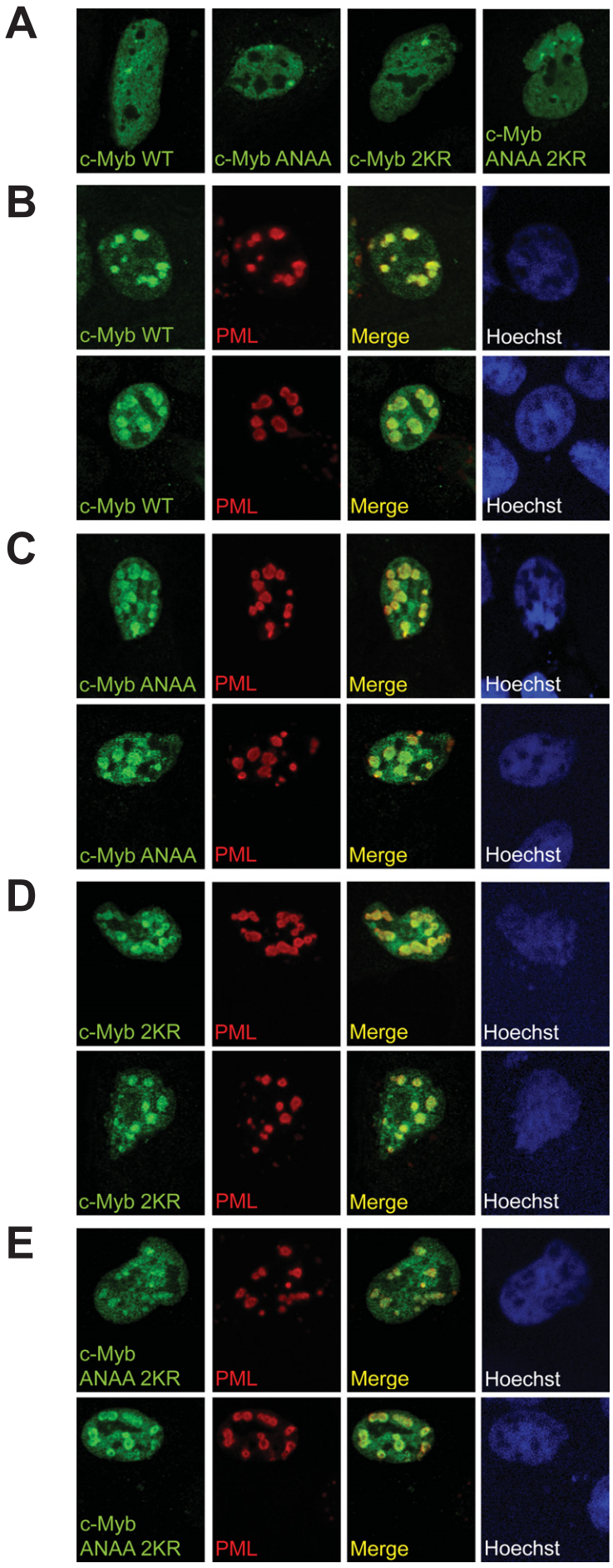

Fold induction (RLU)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PML IVa
- + - + - + - + - +
c-Myb
-
wt
ANAA
2KR
ANAA 2KR

FIGURE 10


A
c-Myb WT
c-Myb ANAA
c-Myb 2KR
c-Myb ANAA 2KR
B
c-Myb WT
PML
Merge
Hoechst
c-Myb WT
PML
Merge
Hoechst
C
c-Myb ANAA
PML
Merge
Hoechst
c-Myb ANAA
PML
Merge
Hoechst
D
c-Myb 2KR
PML
Merge
Hoechst
c-Myb 2KR
PML
Merge
Hoechst
E
c-Myb ANAA 2KR
PML
Merge
Hoechst
c-Myb ANAA 2KR
PML
Merge
Hoechst